Liquid Chromatographic Separation of Enantiomers and Structurally-related Compounds on ß-Cyclodextrin Stationary Phases

by

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ABSTRACT

The retention behaviour of 16 phenothiazines and structurally-related drugs on a ß-cyclodextrin-bonded phase column was studied with respect to pH, mobile phase composition and column temperature. Both isocratic and gradient-elution separations of these compounds were investigated.

The enantiomers of twelve racemic dinitrophenyl amino acid derivatives were separated on a ß-cyclodextrin-bonded phase column. The effects of pH, methanol and triethylammonium acetate (TEAA) buffer concentrations on the retention and resolution were investigated. The chiral recognition mechanism was studied by means of UV-visible, circular dichroism and proton nuclear magnetic resonance spectroscopic methods.

A multiple-interaction type of chiral stationary phase was developed by bonding ß-cyclodextrin to silica gel and modifying the cyclodextrin cavity by flexibly capping its primary hydroxyl or small side. These modified ß cyclodextrin stationary phases contain a hydrophobic cavity, capable of inclusion complexation; aromatic groups, capable of π-π interaction; and polar hydrogen-bonding sites, capable of forming hydrogen-bonding with the polar functional groups of the solutes. These stationary phases exhibit a high stereoselectivity toward a wide variety of chiral compounds. The preparation and properties of these modified ß-cyclodextrin stationary phases are described. The enantiomeric separation of amino acids and their derivatives, of carboxylic acids, of phenothiazine drugs, and of other chiral compounds are reported. The effects of mobile phase composition on the retention and resolution are discussed.

SÉPARATION PAR CHROMATOGRAPHIE LIQUIDE DES ÉNANTIOMORPHES ET DES COMPOSÉS DE STRUCTURES SIMILAIRES AVEC LES PHASES STATIONNAIRES DE ß-CYCLODEXTRINE

ABSTRAIT

La rétention chromatographique de 16 phénothiazines et des drogues de structures similaires sur une colonne de phase adhérée de ß-cyclodextrine a été étudiée en ce qui concerne le pH, la composition de la phase mobile et la température de la colonne. Les séparations isocratiques et d'élution à gradient de ces composés ont été étudiées.

Les énantiomorphes de 12 dérivés au nitrophényl d'acides aminés racémiques ont été séparés grâce à une colonne de phase adhérée de ß-cyclodextrine. L'effet du pH, du méthanol et de la concentration du tampon TEAA sur la rétention et la résolution a été étudié. Le méchanisme de reconnaissance chirale a été étudié par des méthodes spectroscopiques, l'UV-visible, le dichroisme circulaire et la résonance magnétique nucléaire au proton.

Des phases stationnaires chiraux à interactions-multiples ont été développées en faisant adhérer la ß-cyclodextrine au gel de silice et en modifiant la cavité de cyclodextrine en en couvrant flexiblement l'hydroxyl primaire, le petit côté. Ces phases stationnaires modifiées de ß-cyclodextrine ont des cavités hydrophobes qui peuvent former des complexes d'inclusion; des groupes aromatiques capable d'interaction π-π; et des sites polaires pour les liens hydrogène, pouvant donc former des liens de ce type avec les groupes fonctionnels polaires des solutés. Ces phases stationnaires font preuve de stéréosélectivité envers une grande variété de composés chiraux. La préparation et les propriétés de ces phases stationnaires modifiées de ß-cyclodextrine sont décrites. La séparation énantiomorphique des acides-aminés et de leurs dérivés, des acides carboxyliques, des drogues de phénothiazine, et d'autres composés chiraux est présentée. L'effet de la composition de la phase mobile sur la rétention et la résolution est décrit.

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GLOSSARY

CD	circular dichroism
CDP	cyclodextrin-polyvinyl
CDPU	cyclodextrin-polyurethane
CE	capillary electrophoresis
COSY	correlation spectroscopy
CSP	chiral stationary phase
dansyl	1-dimethylaminonaphthalene-5-sulfonyl
dc	direct current
DNP	dinitrophenyl
DP-TFA	2,6-di-O-pentyl-3-O-trifluoroacetyl
ESR	electron-spin resonance
FCD	fiber-optic cyclodextrin-based (sensor)
FDNB	fluoro-2,4-dinitrobenzene
FGF	fibroblast growth factor
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HETP	height equivalent to theroretical plate
HPLC	high performance liquid chromatography
LC	liquid chromatography
MEKC	micellar electrokinetic chromatography
NMR	nuclear magnetic resonance
ORD	optical rotatory dispersion
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
TEAA	triethylammonium acetate
TLC	thin-layer chromatography
UV	ultra-violet

Chapter 1

CYCLODEXTRINS AND THEIR APPLICATIONS IN ANALYTICAL CHEMISTRY

1.1. INTRODUCTION

Cyclodextrins, also known as Schardinger dextrins, cycloamyloses and cycloglucoamyloses, comprise a family of cyclic oligosaccharides obtained from starch by enzymatic degradation. They were discovered in 1891 by Villiers [1], but the first detailed description of the preparation and isolation was made in 1903 by Schardinger [2]. In the preparation process, the starch is treated with a group of amylases called glycosyltransferases or cyclodextrinases. The starch helix is hydrolysed off, and its ends are joined together through α-1,4 linkages [3, 4]. Since these enzymes are not very specific as to the site of hydrolysis, the product contains α-, ß-, and γ-cyclodextrins together with small amounts of higher analogues consisting of up to 13 glucose units [5-8]. Up to now, α-, ß-, γ-, and δ-cyclodextrins, which are comprised of six, seven, eight, and nine glucose units, respectively, have been isolated by selective precipitation with appropriate organic compounds [7-10]. Cyclodextrins with 10 to 13 glucose units were also identified by chromatographic methods [10]. Cyclodextrins composed of less than six glucose units are not known to exist due to steric hindrance [11] and the six-fold character of the starch helix [12].

Investigations of cyclodextrin chemistry have been on the increase for

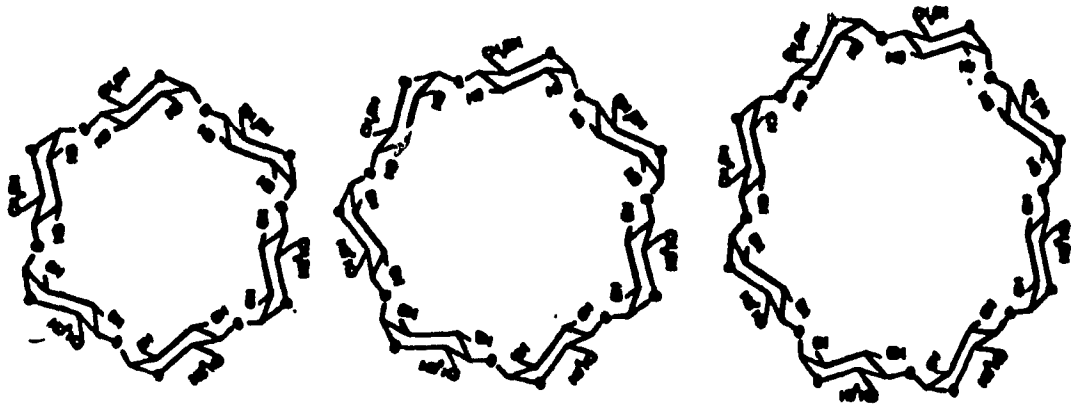
several decades. The descriptions of the structure and properties of cyclodextrins, and their applications have been the subject of several books [9, 13-18], a number of review articles [18-35], more than 800 patents, and innumerable papers. The reasons for the enormous effort in the study of cyclodextrins are that such molecules have inherent interest, that is, their physical and chemical properties merit study; they are the first and probably the most important example of relatively simple organic compounds which exhibit complex formation with other organic molecules; they are excellent models of enzymes which led to their use as catalysts, both in enzymatic and non-enzymatic reactions; and they are natural products and readily available for most researchers.

Cyclodextrins first drew my interest in 1987. After reading Hinze's review article [30] and several chapters in a book edited by Hinze and Armstrong [13], I decided to focus my research on the application of cyclodextrins in chromatographic separations. For the last four years, I have been working mainly on the application of ß-cyclodextrin bonded stationary phases in the separations of enantiomers and structural isomers, and the studies of the separation mechanism. Before presenting the results of my research, I would like to dedicate this first chapter to the descriptions of the structure and the properties of cyclodextrins, and to the reviewing of the applications of cyclodextrins in analytical chemistry.

1.2. STRUCTURES AND PROPERTIES OF CYCLODEXTRINS

1.2.1. Chemical structures

Figure 1.1 shows the chemical structures of α-, ß-, and γ-cyclodextrins. As their appearance suggests, in the cyclodextrin molecules the glucose units,



α-cyclodextrin ß-cyclodextrin γ-cyclodextrin

Figure 1.1. Structure of α-, ß-, and γ-cyclodextrin.

all in classical C1 chair conformation, are linked by α-1,4 bonds. This geometry gives the cyclodextrin the overall shape of a truncated cone with the wider side formed by the secondary 2-, and 3- hydroxyl groups and the narrower side by the primary 6-hydroxyl (Figure 1.2). The number of glucose units determines the dimension and size of the cavity (Figure 1.3). The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges. The non-bonding electron pairs of the glycosidic oxygen bridges are directed towards the inside of the cavity, producing a high electron density and lending it some Lewis-base character. As a result of this special arrangement of the functional groups in the cyclodextrin molecules, the cavity is relatively hydrophobic compared to water while the external faces are hydrophillic. In the cyclodextrin molecules, a ring of hydrogen bonds is also formed intramolecularly between the 2-hydroxyl and the 3-hydroxyl groups of adjacent glucose units. This hydrogen-bonding ring gives the cyclodextrin a remarkably rigid structure.

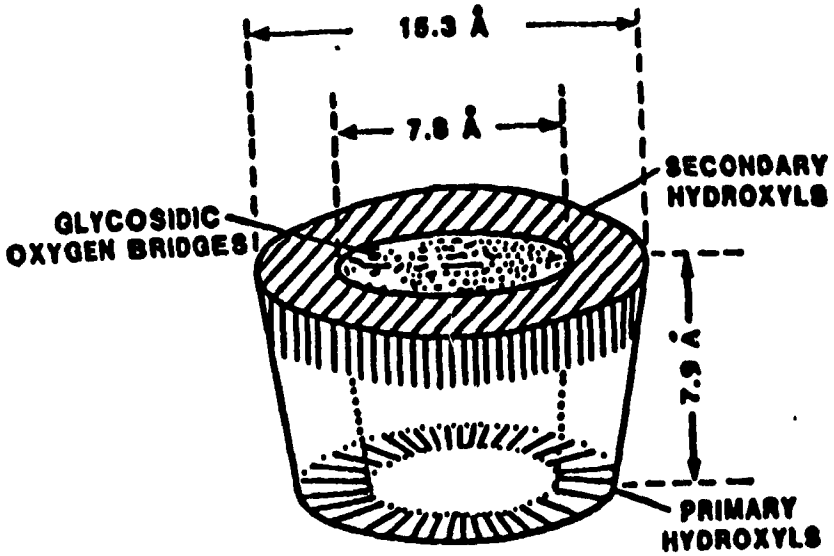
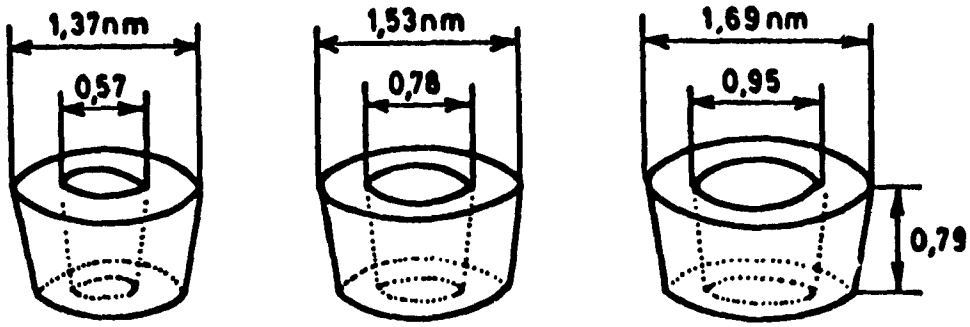


Figure 1.2. Functional structural scheme of ß-cyclodextrin.



α-cyclodextrin ß-cyclodextrin γ-cyclodextrin

Figure 1.3. Molecular dimensions of cyclodextrins.

1.2.2. The properties of cyclodextrins

As a consequence of these structural features, cyclodextrins have some unique physical and chemical properties. Some of the important physical properties and characteristics are listed in Table 1.1.

Cyclodextrins are water-soluble with solubilities of 14.5, 1.85, and 23.2 g/100ml for α-, ß- and γ-cyclodextrin, respectively. The spectroscopic studies on cyclodextrin in aqueous solution suggest that the conformation of cyclodextrins in solution is almost identical to their conformation in the crystalline state.

Table 1.1. Charateristics of α-, ß-, and γ-Cyclodextrins

Characteristics	α-	ß-	γ
No. of glucose units	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100 mL)	14.5	1.85	23.2
Cavity diameter (Å)	4.7 - 5.3	6.0 - 6.5	7.5 - 8.3
Height of torus (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
pKa values	12.33	12.20	12.08

Cyclodextrins are stable in alkaline solutions. However, they are susceptible to acid hydrolysis. Partial acid hydrolysis of cyclodextrins produces glucose and a series of acyclic malto-saccharides [18]. The stability of cyclodextrins against acid hydrolysis depends on the temperature and acidity. For example, the rate constants for the hydrolysis of ß-cyclodextrin at 100°C in the solutions of 0.0115 N HCl and 1.15 N HCl are 1.3×10^{-4} and 4.8×10^{-2} min^{-1}, respectively. In the presence of 1.15 N HCl, the rate constants at 40°C and 80°C are 1.0×10^{-5} and 3.7×10^{-3} min^{-1}, respectively. Under normal experimental conditions (pH higher than 3.5, and temperature lower than 60°C), cyclodextrins are fairly stable.

Although the cleavage of the 1,4-glycosidic bonds can occur on γ-irradiation of crystalline ß- and γ-cyclodextrins [18], they are fairly resistive to the light within UV-visible and IR ranges.

The most characteristic property of cyclodextrins is their remarkable ability to form inclusion complexes with a wide variety of guest molecules ranging from organic or inorganic compounds of neutral or ionic nature to noble gases. It seems that the only obvious requirement is that the guest molecules must fit into the cavity, even if only partially. Complex formation in solution is a dynamic equilibrium process which can be illustrated by equation (1),

$$CD + G \rightleftharpoons CD\text{-}G \qquad (1)$$

where CD is cyclodextrin, G is the guest molecule and CD-G is the inclusion complex. The stability of the inclusion complex can be described in terms of a formation constant (K_f) or a dissociation constant (K_d) as defined in equation (2) and equation (3), respectively.

$$K_f = [CD\text{-}G]/([CD]\cdot[G]) \qquad (2)$$

$$K_d = 1/K_f = ([CD]\cdot[G])/[CD\text{-}G] \qquad (3)$$

It has been generally accepted that the binding forces are involved in the complex formation are: (i) van der Waals interactions (or hydrophobic interactions) between the hydrophobic moiety of the guest molecules and the cyclodextrin cavity; (ii) hydrogen bonding between the polar functional groups of guest molecules and the hydroxyl groups of cyclodextrin; (iii) release of high energy water molecules from the cavity in the complex formation process; and (iv) release of strain energy in the ring frame system of the cyclodextrin. The role of hydrogen bonding is not universal as stable complexes are formed with guests such as benzene which cannot form hydrogen-bonds.

Regardless of what kind of stabilising forces are involved, the geometric capability and the polarity of the guest molecules, and the medium are the most important factors for determining the stability of the inclusion complex. Geometric rather than the chemical factors are decisive in determining the kind of guest molecules which can penetrate into the cavity. If the guest is too small, it will easily pass in and out the cavity with little or no bonding at all. Complex formation with guest molecules significantly larger than the cavity may also be possible, but the complex is formed in such a way that only certain groups or side chains penetrate into the cyclodextrin cavity.

Stability of an inclusion complex also depends on the polarity of the guest molecule. Only substrates which are less polar than water can form inclusion complexes with cyclodextrins. The stability of a complex is proportional to the hydrophobic character of the guest molecule. Highly hydrophillic molecules complex very weakly or not at all.

In principle, inclusion complexes can be formed either in solution or in the crystalline state. However, complexation is usually performed in the presence of water. The stability strongly depends on the nature of the medium used for complexation. Although inclusion complex formation takes place in an organic solvent, the guest molecules are generally only weakly complexed.

Complexing ability can also be improved by chemically modifying the cyclodextrin molecules. Cyclodextrins can be modified by (i) substituting for the

H-atom of the primary or secondary hydroxyl groups, (ii) substituting for one or more primary and/or secondary hydroxyl groups, (iii) eliminating the hydrogen atoms of the -CH_2OH groups (e.g. by conversion to -COOH), or (iv) splitting one or more C_2-C_3 bonds through a periodate oxidation. Recent interest in the use of chemically modified cyclodextrins for various purposes has generated a number of reviews dedicated to the syntheses and application of cyclodextrin derivatives [57-59]. In several other reviews [15, 18, 31], some information on cyclodextrin derivatives has also been included.

As a result of complex formation, the characteristic properties of the included substance, such as solubility [36, 37], chemical reactivity [20, 38], pKa values [39, 40], diffusion [15, 41], electrochemical properties [42-45], and spectral properties [46-56] will be changed. This unique property has led cyclodextrins to a widespread utilization in pharmaceutical, food, chemical and other industries [18]. In the pharmaceutical industry, cyclodextrins and their derivatives have been used in drugs either for complexation or as auxiliary additives such as solubilisers, diluents, or tablet ingredients to improve the physical and chemical properties, or to enhance the bioavailability of poorly soluble drugs [18, 60-62]. In food, cosmetics, toiletric and tobacco industries, cyclodextrins have been widely used either for stabilisation of flavours and fragrances or for elimination of undesired tastes, microbiological contaminations, and other undesired components [63-66]. In the chemical industry, cyclodextrin and their derivatives are used as catalyses to improve the selectivity of reactions, as well as for the separation and purification of industrial scale products. It has been reported that up to the end of 1986, about 750 patents were published relating to cyclodextrins and their applications, and with an increase at the rate of 80 per annum. It is expected that with increasing production, broadening research and decreasing prices, the applications of cyclodextrins and their derivatives will rapidly increase in a wide of variety of industries. More details on the application of cyclodextrins in industry can be obtained in recent monographs [15, 18]. In recent years, cyclodextrins and their derivatives have also been used in various field of analytical chemistry, especially in analytical separations. This will be the topic

of next section.

1.3. APPLICATIONS OF CYCLODEXTRINS IN SPECTROMETRIC METHODS

The high density prevailing inside the cyclodextrin cavity can mobilize the electrons of the included guest molecules, resulting in changes in various spectral properties of both the guest and cyclodextrin itself [67]. The effect of cyclodextrins on the spectral properties of guest molecules has led to their use as reagents in various spectrometric analyses, including UV-visible spectrophotometric analysis, fluorescence and phosphorescence methods, and nuclear magnetic resonance spectroscopy.

1.3.1. Cyclodextrins in UV-visible spectrophotometric analysis

Since the spectral changes of coloured molecules in the presence of cyclodextrins was first observed in 1951 by Cramer [68], the effect of cyclodextrins on UV and visible spectra of various guest molecules has been studied [46, 47, 69, 70]. Figure 1.4 shows the UV spectra of Amphotericin B in water and in aqueous γ-cyclodextrin solutions. Generally, a bathochromatic shift and an absorbance change (increase or decrease) can be observed in the presence of cyclodextrins. The changes in absorbance upon adding cyclodextrins have been used to calculate the dissociation constants using the Scott equation [71] or the Benesi-Hildebrand equation [72].

The complexation of analyte and/or colouring reagent can effectively change their properties. Some of the most useful effects are as follows: (i) increased solubility of apolar analytes and/or reagents in aqueous media; (ii) increased stability of sensitive reagents and the colour complexes in aqueous or non-aqueous solutions; (iii) increased sensitivity of the colour reactions

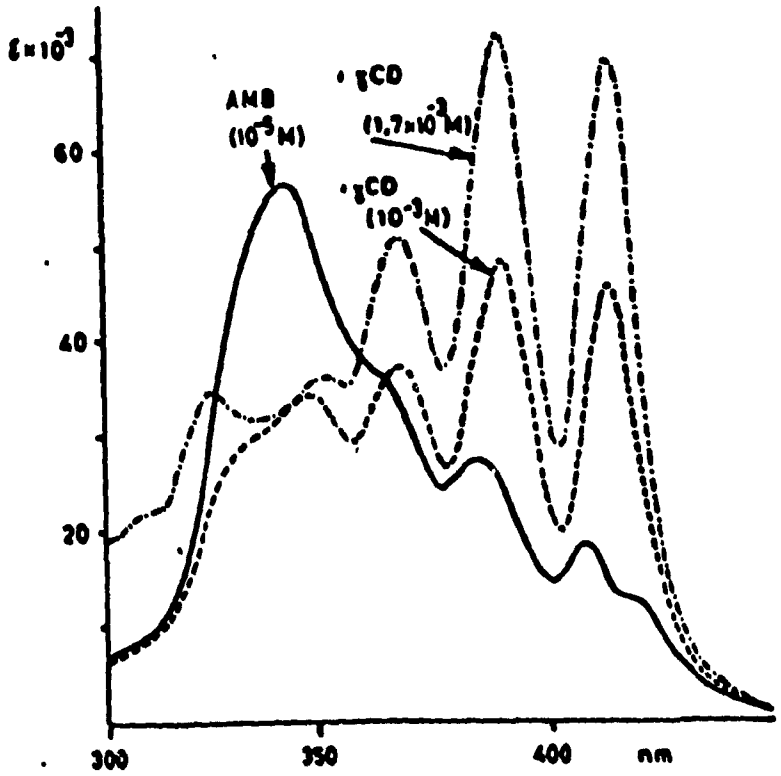


Figure 1.4. UV spectra of Amphotericin B in water and in aqueous γ-cyclodextrin solution.

through intensification of UV absorption; (iv) improved selectivity of colour reactions. These effects make cyclodextrins useful auxiliaries in the spectrophotometric determinations of a wide variety of compounds and elements.

The effect of ß-cyclodextrin on colour reactions of various metal ions with triphenylmethane, xanthene acid dyes and some other colouring reagents has been studied by Qi *et al.*[73] It was found that selectivity of the colour reactions is improved by adding ß-cyclodextrin in the solution. Recently, Huang *et al.*[74] studied the effect of ß-cyclodextrin on the association compound system of metal (Mo, Zn, Co)-thiocyanate basic dyes (Malachite green, crystal

violet, Rhodamine B, Rhodamine 6G, and Butylrhodamine B). The presence of ß-cyclodextrin resulted in a more sensitive and stable system. The improved sensitivity and stability resulted from the formation of ß-cyclodextrin inclusion complexes with the basic dyes, thus increasing the solubility of the basic dyes and creating a favourable microenvironment for the colour reactions. Tao *et al.* [75] reported that in the spectrophotometric determination of copper in leaves and human hair, the sensitivity of colour reaction of Cu(II) and meso-tetrakis (4-methoxy-3-sulfophenyl)porphyrin was enhanced by 50% in the presence of α-cyclodextrin.

ß-Cyclodextrin can form a 1:1 inclusion complex with 1,2-aminoanthraquinone in aqueous solution. This is employed to solubilize the anthraquinone in water for use as a ligand for metal ions. In the presence of ß-cyclodextrin, 1, 2-diaminoanthraquinone has been used for the determination of palladium at trace levels by spectrophotometry [76]. The limit of detection of 11 ng/ml can be obtained.

Zhe *et al.* [77] described a new spectrophotometric method for the determination of microamounts of Zn based on the Zn-dithizone colour reaction sensitized with ß-cyclodextrin. The apparent molar absorptivity at 538 nm is 8.37 times as large as that in the absence of ß-cyclodextrin.

Cyclodextrins can be used as stabilizers for colouring compounds and colour indicators used in analytical chemistry. Sakata *et al.* [78] used α-cyclodextrin as a stabilizer to increase the stability of indicators used for the spectrophotometric determination of hydrogen peroxide in body fluids.

Cyclodextrins and their derivatives have also been used in enzyme assays and enzyme activity measurements. Modified cyclodextrins, glucosyl-α-cyclodextrin and maltosyl-α-cyclodextrin have been used in an analytical system to increase the accuracy and sensitivity of the assay of amylase [79]. In the amylase detection procedure, the sample is treated with a reagent mixture containing benzilidene-p-nitrophenyl-maltoptaoxide, glucoamylase, glucosyl-α-cyclodextrin and some other components. The mixture was monitored spectrophotometrically at 405 nm.

γ-Glutamyl transpeptidase activity can be spectrophotometrically

determined using L-γ-glutamyl-p-nitroanilide as substrate in the presence of sulfopropyl ß-cyclodextrin in the reaction solution [80]. Addition of the modified ß-cyclodextrin to the reaction solution enhances the solubility of the substrate, thus increasing the sensitivity of the measurement.

Up to now, in UV-visible spectrophotometric analysis cyclodextrins are mainly used as reagents to improve the solubility and stability of coloured complexes formed between analyte and colouring agents, and to enhance the sensitivity and selectivity of colouring reactions. With broadening research in this field, more applications of cyclodextrins and their derivatives in UV-visible spectrophotometric analysis are expected.

1.3.2. Cyclodextrin in analytical luminescence spectrometry

Molecular luminescence spectrometry, especially molecular fluorescence spectrometry, has become established as a routine technique in many analytical applications. In many cases, molecular luminescence spectrometry can yield a lower detection limit and greater selectivity than molecular absorption spectrometry. However, although most compounds show strong fluorescence or phosphorescence in organic solvents, the intensity of luminescence is rather weak in water. Adding cyclodextrins, which form inclusion complexes with analyte molecules in aqueous solution, can result in significant enhancement of the fluorescence or phosphorescence. The inclusion of analyte molecules into the cyclodextrin cavity can offer certain advantages:

1. The structural conformation of the cyclodextrin protects the fluorescing singlet state or the phosphorescing triplet state of the analytes from external quenchers [81-84].

2. As a consequence of inclusion complex formation, the rotation of the guest molecule is hindered, and the relaxation of the solvent molecules is considerably decreased. Both of these effects can result in a decrease in the vibrational deactivation.

3. The cyclodextrin cavity behaves similarly to the organic solvent. It affords an apolar surrounding for the included chromophore. This altered microenvironment can provide favourable polarity and acid/base equilibria for enhanced quantum efficiencies and hence the intensities of luminescence.

4. The cyclodextrin solution can improve the detection limit for hydrophobic analytes in aqueous solution by increasing their solubility or for hydrophillic analytes by increasing solubility of the water-insoluble fluorescent compounds into which the analytes are incorporated.

Inclusion complex formation with cyclodextrin usually results in a higher fluorescence quantum yield. It has been found that the fluorescence intensities of many compounds, such as pyrene [85], various illicit drugs, narcotics, hallucinogenics [86], and polychlorinated biphenols [87] are significantly increased by the complex formation with cyclodextrins and their derivatives. 1-Anilinonaphalene-8-sulphoate is strongly fluorescent in organic solvents, but shows only a negligible fluorescence in aqueous solution. However, in an aqueous cyclodextrin solution the fluorescence becomes significant. The fluorescence intensity of this compound in ß-cyclodextrin solution is increased about ten-fold [69]. The effects of aqueous cyclodextrins on the fluorescence emission of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate-labeled glutathione, acetylcysteine and cysteine and of some dansylated amino acid were recently investigated by Baeyens *et al.* [88] In the presence of cyclodextrin, fluorescence enhancements up to 8-fold were observed for these compounds in comparison with the original values.

The fluorescence intensity of naphthalene in aqueous solution decreases upon aeration. In the presence of a water-soluble sulphopropylated ß-cyclodextrin the quenching of naphthalene by aeration is totally supressed [89]. A recent study shows that both monomer and excimer fluorescence of 1, 3-di(α-naphthyl) propane can be quenched by RNA in methanol-water binary solvents. The quenching, however, is hindered in the presence of ß-cyclodextrin

[90].

Similarly, the quenching of halonaphthalene phosphorescence in water by $NaNO_2$ can be substantially inhibited by ß-cyclodextrin [91]. The rate of inhibition depends on the tightness of the fit of the analyte into the cyclodextrin cavity or the ratio of cavity to guest diameter [92].

Retinal, which is normally insoluble in water and is not fluorescent in solution at room temperature, emits luminescence in the region of 450 nm, permitting fluorescence detection when complexed with ß- or γ-cyclodextrin, even in air-saturated aqueous solution [93].

Cyclodextrins have also been used in the luminescence detection of volatile compounds. Filter paper, treated with cyclodextrin, is capable of trapping the volatile compounds, such as 1- and 2-naphthol, efficiently on filter paper and permitting a strong luminescence signal to be observed [94]. Cyclodextrins can be used as solid matrices for obtaining room-temperature fluorescence (RTF) and room temperature phosphorescence (RTP) from the absorbed compounds. A 30:70 ß-cyclodextrin/NaCl mixture produced strong luminescence signals from absorbed compounds without need for a heavy atom. This matrix provides a sensitive method for the determination of p-aminobenzoic and phenanthrene [95].

In most cases, the presence of cyclodextrin will enhance the luminescence. However, cyclodextrin can also selectively quench the luminescence of some compounds if the chromophore and the quencher are included in the same cavity [81]. A study of the effect of ß-cyclodextrin on the fluorescence of xanthene dyes, coumarins and pyromethene-difluoroboron complexes in aqueous solution shows that the presence of ß-cyclodextrin enhances the fluorescence of 7-hydroxycoumarin and coumarin 102 and 105, but quenches the fluorescence of the 7-hydroxy-4-methylcoumarin salts [96]. This behaviour of cyclodextrins provides a new approach to multicompoent fluorometric analysis.

Biologically active amines, amino acids, peptides, catecholamines, steroidal compounds, etc. can be luminescently determined as their dansyl derivatives. The derivatization procedure are usually carried out in aqueous

medium ($NaHCO_3$ solution). Prior to the determination the dansyl derivatives must be transferred from aqueous solution into an apolar medium, to allow for for stronger luminescence. This time-consuming procedure has been replaced by using a host-guest sensory system of dansyl-modified ß-cyclodextrin [97]. This system shows high sensitivities for steroidal compounds.

Recently, a fibre-optic cyclodextrin-based (FCD) sensor for fluorometric detections of a wide variety of organic compounds was developed by Alarie and Vo Dinh [98]. This FCD sensor uses laser excition and fluorescence detection with ß-cyclodextrin immobilized at the tip of an optical fibre. The sensitivity of this FCD sensor is 14 times as great as that of a bare optical fibre when measurement was made for pyrene with the sensor immersed in a buffer after a 10-min incubation period.

1.3.3. Cyclodextrins in NMR spectroscopy

^1H NMR spectra of cyclodextrins and their inclusion complexes were first investigated by Demarco and Thakkar [99, 100]. These authors found that when the aromatic moiety of a guest molecule is included in the cyclodextrin cavity, protons located within the cavity (3-H and 5-H) are susceptible to anisotopic shielding be the aromatic moiety, thus a upfield shift is observed. Protons located on the exterior of the cavity (2-H, 4-H, and 6-H) are relatively unaffected. Following this pioneering work, NMR spectroscopy became the most powerful tool for the study of inclusion complex formation between cyclodextrins and a variety of guest molecules. Initially, the investigations were only carried out in solution by ^1H NMR, but now, ^{13}C NMR [101], ^{15}N NMR [102], ^{19}F NMR [103] and ^{31}P NMR [104] spectroscopic methods all have been used for the inclusion complex formation studies, even in solid state.

In NMR spectroscopic analysis, cyclodextrins are mainly used as chiral NMR shift reagents. In many cases, the influence of cyclodextrin inclusion complex formation on the NMR features of the two enantiomers of a chiral compound differ in chemical shifts [105]. A ^{19}F NMR study [103] of the

formation of diastereoisomeric inclusion complexes between fluorinated amino acid derivatives and α-cyclodextrin in 10% D_2O solution shows that the chemical shifts of the R-amino acid derivatives-α-cyclodextrin inclusion complexes are upfield from those of their S-analogues for deprotonated N-(p-fluorobenzoyl)valine, deprotonated α-(p-fluophenyl)glycine and N-acetyl-α-(p-fluorophenyl)glycine. The shift difference between the diastereoisomers formed with R- and S- (or D- and L-) enantiomers can be used for chiral analysis, and optical purity determinations. For example, the interaction of ß-cyclodextrin with propanolol hydrochloride produces diastereomeric pairs. Observed in D_2O solution at 400 MHz, the protons of the antipode give ^1H NMR signals which differ in chemical shifts. The intensity of the resonance signals for each diastereoisomers has been used for optical purity determination [106]. By adding racemate to pure (-)-isomer, this novel technique is able to measure optical purity of propanolol hydrochloride in water down to the level of 1%.

1.4. CYCLODEXTRINS IN ELECTROCHEMICAL ANALYSIS

1.4.1. Electrochemical behaviour of cyclodextrins and cyclodextrin inclusion complexes.

Cramer [107] reported in 1953 that adding cyclodextrin to an aqueous methylene blue solution resulted in an increase of its redox potential by 0.043-0.048 V at pH 7.0 and 8.0, respectively. Following Cramer's investigation much work has been devoted to the study of the electrochemical behaviour of cyclodextrins and cyclodextrin inclusion complexes, and to the utilisation of various electrochemical methods, such as cyclic voltammetry, polarography, potentiometry and conductometry, for the measurements of stability constants and dissociation rate constants of cyclodextrin inclusion complexes [108-116]. In a review paper, Bersier *et al.* [112] described the recent development of the

electrochemistry of cyclodextrins and cyclodextrin inclusion complexes.

Cyclodextrins, which do not form dc (direct current) polarographic waves, exhibit adsorption/desorption peaks on cyclic voltammograms, demonstrating adsorption processes [117-121]. The surface tension of mercury is lowered by the absorption of cyclodextrins or their complexes, and the drop time of the mercury decreased in cyclodextrin solutions [117]. Detailed investigations indicate that the absorption of cyclodextrins depends on the electrode potential applied, and shows a very complicated character due to two-dimensional condensation of cyclodextrins and reorientation effects in the adsorbed state [120]. At less negative potential, the cyclodextrin molecules are oriented with the cavity perpendicular to the electrode surface, while at more negative potential, orientation is intermediate between "parallel" and "perpendicular".

Adsorption effects have been exploited for the quantitative assays of cyclodextrins. Yamaguchi *et al.* [122] studied the effect of cyclodextrins on the polarographic oxygen waves for the quantitative determination of trace amounts of α- and ß-cyclodextrins. An indirect polarographic method based on the ability of cyclodextrin to form complexes with linoleic acid has been developed by Laakso *et al.* [123]. The method has been applied to the analysis of immobilized cyclodextrins as well as cyclodextrins in complex mixtures of starch and starch-degrading enzymes.

The formation of inclusion complexes can result in dramatic changes in the electrochemical properties of guest molecules. Jones and Parr [114] studied the effect of ß-cyclodextrin on the peak height and half-wave potential of the polarographic reduction of methyl, ethyl, propyl and butyl hydroxybenzoates. Inclusion complex formation with ß-cyclodextrin causes a decreased peak height and a shift of the $E_{1/2}$ toward negative potential for each of the esters of hydroxylbenzoic acid. The changes in potential were observed in the following order: ethyl > propyl > butyl. This was a result of the electron redistribution due to the formation of inclusion complexes, and reflected the tendency of these esters to complex with ß-cyclodextrin. Figure 1.5 shows the differential pulse polarograms of benzyl viologen in phosphate buffer without

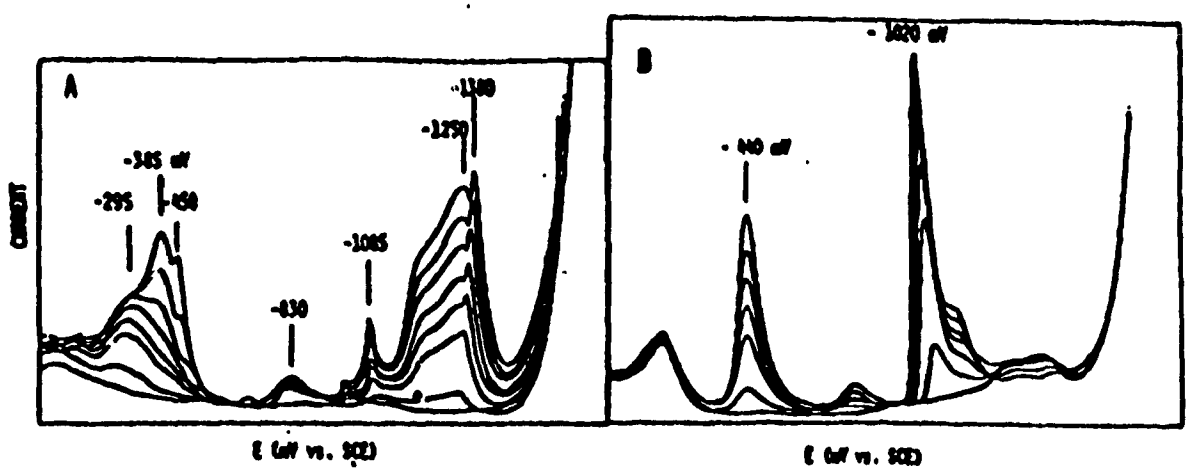


Figure 1.5. Differential pulse polarograms of benzyl viologen in phosphate buffer (pH 7.0) (A) without and (B) in the presence of 0.01 M ß-cyclodextrin. Depolarizer concentration, 2.4 - 18.8 µg/ml of polarographic solution. Pulse amplitude, -25 mV; scan rate, 5 mV/s; T, 23 ± 0.5° [112].

(A) and in the presence of ß-cyclodextrin (B) [112]. The compexitiy of the benzyl viologen polarography makes the polarographic assay difficult. However, in the presence of cyclodextrin a much simpler differential pulse polarogram is observed.

These studies and some other investigations [115, 124-128] suggest that polarography and voltammetry are suitable for studying the inclusion phenomenon of cyclodextrins with electroactive molecules in aqueous solution. From the changes in peak height and in half-wave potential, both the stability constants and the diffusion coefficients of the inclusion complexes can be detected by polarography and voltammetry [129-133]. Electrochemical methods may prove to be powerful techniques in further elucidating the nature of the inclusion complexes.

1.4.2. Use of cyclodextrin in the electrochemical analysis

Relatively few reports have been published on the use of cyclodextrins in electrochemical analysis as compared with their use in chromatographic separations. Recently, some attempts have been made to use the enhanced selectivity resulting from cyclodextrin inclusion complex formation for the polarographic/voltammetric analysis of electroactive guests.

Matsue *et al.*[134] have developed a regioselective electrode system with a poly(perfluorosulfonic acid)-coated electrode based on cyclodextrin complexation for the determination of o-nitrophenol in the presence of p-nitrophenol. The p-nitrophenol shows an extraordinary small reduction peak on a regioselective electrode in α-cyclodextrin solution, while the effect of α-cyclodextrin on o-nitrophenol is small. The system is 33 times more sensitive to o-nitrophenol than to p-nitrophenol, thereby allowing an accurate determination of o-nitrophenol in the presence of its p-isomer. Species-selective voltammetric determination of o-nitrobenzene derivatives was also successfully performed on this electrode system with α-cyclodextrin in solution [135].

Voltammetric sensors responsive to anionic guests, based on host-guest molecular recognition, have recently been developed by Nagase *et al.* [136]. These voltammetric sensors were constructed with membrane assemblies of lipophilic cyclodextrin polyamine containing anion receptors deposited directly on glassy carbon electrodes by the Langmuir-Blodgett (LB) method [137]. Macrocyclic polyamine and cyclodextrin polyamine is capable of binding with anionic guests in multiprotonated forms. The response to the anionic guests appears as the decrease of peak height in cyclic voltammetry using $[Fe(CN)_6]^{4+}$ as marker ion. The selectivities for positional isomers of phthalate were found in the order of m-isophthalate > p-terephthalate > o-phthalate. The selectivity observed is possibly due to the host-guest interaction involving in the cyclodextrin cavity.

Tamagaki *et al.* [138] described the response of gold electrodes coated with a monolayer of cyclodextrin-thio derivativies. The electrochemical

behaviour of these electrodes has been studied voltammetrically using ferrocenecarboxylic acid, $Fe(CN)^{4-}$ and Fe^{2+} as the marker electroactive substrate.

Several gases can form inclusion complexes with cyclodextrins in the solid state when treated with cyclodextrin in the presence of gas. In solution, such complexes are dissociated. This could be a new approach for the quantitative determinations of the gases. Matre *et al.* [139] have used cyclic voltammetry for the assay of oxygen released from α-cyclodextrin.

1.5. APPLICATIONS OF CYCLODEXTRINS IN CHROMATOGRAPHIC SEPARATIONS

In recent years, cyclodextrins and their derivatives have received much attention in the field of chromatographic separations. The wide interest in the use of cyclodextrins as a separation medium arises from the fact that cyclodextrin can offer a highly selective system for chromatographic separation. Cyclodextrin complexation is highly selective, moreover stereoselective. Inclusion complex formation is mainly affected by the hydrophobicity and the shape of guest molecules. Thus, steric factors are crucially important for the formation and the stability of cyclodextrin inclusion complexes. The partitioning and binding of many hydrophobic and hydrophillic organic molecules to the cyclodextrin cavity can be much more selective than the partitioning and binding to a single solvent or to a single traditional stationary phase. For this reason, cyclodextrins find their use in typically difficult separations of enantiomers, diastereomers, structural isomers, and geometric isomers, in all current types of chromatography [18,35].

1.5.1. Cyclodextrin in thin-layer chromatography

Cyclodextrins and their derivatives have been used for the thin-layer chromatographic (TLC) separations of a great variety of compounds. In TLC, cyclodextrins are mainly used as components of the mobile phases to improve the selectivity, or to enhance the chromatographic detection.

Aqueous α-cyclodextrin solution has been applied as mobile phase additive for the separation of a wide variety of substituted aromatic compounds. Hinze *et al.* [140, 141] have reported the separation of 25 phenols and naphthols, and 18 substituted benzoic acid derivatives on polyamide plates with α-cyclodextrin in the mobile phase. It was found that in a given family of compounds, for example, o-, m-, and p-nitrophenols, the isomer with the largest stability constant for its α-cyclodextrin complex had the larger R_f value. In general the order of R_f is: p- > m- > o-substituted isomer. The application of α-cyclodextrin is limited by its narrow cavity diameter. Larger molecules do not fit the cavity, thus the selectivity is not improved for those larger molecules.

ß-Cyclodextrin, which has a larger cavity diameter, shows a wider application in TLC separations. Lepri *et al.* [142] recently reported the separation of methylthiohydantoin derivatives of DL amino acids and a number of naphthyl derivatives by TLC on SiLC18-50F plates using aqueous ß-cyclodextrin solution as mobile phase. The enantiomeric separation of dansyl-, dinitrophenyl-, dinitropyridyl- and α-naphthylamide-substituted amino acids has been achieved on the layer of SiLC 18-50F plates developed with aqueous-organic solution containing ß-cyclodextrin as chiral agent [142a]. Armstrong *et al.* [143] reported the resolution of a wide variety of racemic compounds by reversed-phase TLC with mobile phases containing highly concentrated solution of ß-cyclodextrin. The separated chiral compounds include the drug labetalol and mephytoin, methallocenes, crown ethers, methyl-p-toluenesulfinate, nornicotine derivatives, and several dansyl- and ß-naphthylamide-substituted amino acids.

An obvious limitation to the use of native ß-cyclodextrin as mobile phase

additive is its low aqueous solubility. Highly water-soluble cyclodextrin polymers and derivatives have overcome this limitation and proved to very useful in the TLC separation of a wide variety of compounds. The reversed-phase TLC behaviour of various compounds, such as 17 substituted s-triazine derivatives [144], 21 bartiturates [145], 25 triphenylmethane derivatives and analogues [146] and 33 nitrostyrene derivatives [147] have been studied on silica or cellulose plates in the presence of water-soluble ß-cyclodextrin polymers. Recently, Duncan and Armstrong [148] reported the enantiomer separations of amino acid derivatives and alkaloids by TLC on different types of reversed-phase plates with a mobile phase containing maltosyl-ß-cyclodextrin. Partially substituted hydroxypropyl- and hydroxyethyl-ß-cyclodextrins have also proved to be effective chiral mobile phase additives for the TLC enantiomeric separation of various chiral compounds, including dansyl- and ß-naphthylamide amino acids [149]. Hinze *et al.* [150] reported the resolution of isomeric o-, m-, and p-substituted benzenes, pesticide, polycyclic aromatic hydrocarbon, and drug test mixtures by TLC on a polyamide stationary phase with an aqueous solution of urea-solubilized ß-cyclodextrin as mobile phase.

In the TLC assessment of drug purity, on-plate decomposition of drugs can occur, resulting in artifacts. To overcome this on-plate degradation, Grinberg *et al.* [151] used aqueous γ-cyclodextrin solution as the spotting solution followed by a mobile phase containing hexadecyltrimethylammonium bromide as a micelle generator. The inclusion complex formation between γ-cyclodextrin and the drug molecules successfully prevented degradation during separation procedure.

Highly selective cyclodextrin-bonded silica gels has also been developed by Armstrong [152] for the use as stationary phases in TLC and HPLC. The separation of enantiomers, diastereomers, and structural isomers have been achieved by using these cyclodextrin-bonded stationary phases [153].

1.5.2. Cyclodextrin in affinity chromatography

Cyclodextrins are known to inhibit some enzymes. Immobilized cyclodextrin can be used in artificial affinity column chromatography.

α-Cyclodextrin competitively inhibits ß-amylase. An α-cyclodextrin-Sepharose column developed by coupling α-cyclodextrin to Sepharose 6B at pH 13 can be used to separate ß-amylase from α-amylase and albumin [154]. The α-amylase and albumin are not retarded and passed through the column. The ß-amylase is then eluted by adding α-cyclodextrin to the starting buffer, thus, separating it from other enzymes and proteins. The activity of the eluted ß-amylase is higher owing to its purification. The α-cyclodextrin-Sepharose affinity column has been used to recover Chalara-Paradoxa amylase from the saccharified starch solution for repeated use [155].

Similarly, ß-cyclodextrin is an affinity ligand for cereal α-amylase. Thus, a ß-cyclodextrin column can be used to separate α-amylase from ß-amylase and other enzymes [156]. ß-Cyclodextrin also shows strong affinity to spinach leaf starch-debranching enzymes. Therefore, the ß-cyclodextrin-bound Sepharose 6B column can be used to purify the spinach leaf starch-debranching enzyme [157]. The column loaded with spinach leaf is washed with sodium acetate buffer to remove other enzymes. When the effluent is free from material absorbing at 280 nm, the ß-cyclodextrin solution is used to release the retarded starch-debranching enzyme. In fact, ß-cyclodextrin has been shown to be an affinity ligand for all types of amylolitic enzymes [158], but with different affinity. The enzymes which are retarded on the ß-cyclodextrin affinity column are eluted by using different concentrations of ß-cyclodextrin.

ß-Cyclodextrin tetradecasulfate has a very strong affinity to fibroblast growth factor (FGF). A biaffinity chromatographic system with a stationary phase of the ß-cyclodextrin-tetradecasulfate polymer mixed with Cu-Sepharose has been used for the purification of FGF [159]. Basic FGF can be purified by about 200,000-fold from rat chondrosarcoma.

A ß-cyclodextrin column capable of double recognition (carbonyl

recognition and hydrophobic recognition) has been used in affinity column chromatography [160]. The packing material is prepared by immobilizing the primary A,D-bis(2-aminoethyl)sulfenyl-capped ß-cyclodextrin on the acryonitrile-methyl acrylate copolymer via a amide linkage. A packed column of 2.7 cm in length can be used for the affinity chromatographic separation of any guest molecule having a hydrophobic site and a carbonyl group from other compounds of similar structures.

1.5.3. Cyclodextrins in electrophoresis

In 1982, Tazaki *et al.* [161] first effectively demonstrated the usefulness of cyclodextrins in the isotachophoretic analysis of alkali and alkaline metals. The authors found that the use of α-cyclodextrin as a complexing agent improved the separation through a host-guest interaction. Since that time several other groups have become active in the investigation of using cyclodextrins in various types of electrophoresis, and the last four years have seen many advances in this field.

In capillary zone electrophoresis, cyclodextrins have been successfully used as additives in the carrier system for the separation of structural isomers and structurally related compounds. The capillary electrophoretic separation of 9 plant growth regulators using a mixed carrier system containing ß-cyclodextrin modifier was recently reported by Yeo *et al.* [162]. The results showed that all the plant growth regulators were satisfactorily separated within 20 min (see Figure 1.6).

As chiral recognition agents, the use of cyclodextrins in the carrier system has made capillary zone electrophoresis a useful technique for the enantiomeric separation of a wide variety of chiral compounds, such as terbutaline and propranolol [163], dansyl-DL-amino acids [164], DL-tryptophan and (±)-epinephrine [165], and epkedrine norephedrine, norepinephrine, and isoproterenol [166].

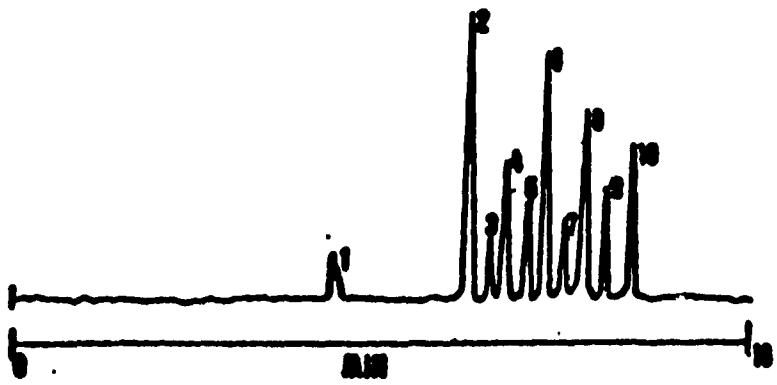


Figure 1.6. Electropherograms for the nine plant growth regulators. Electrophoretic conditions: 0.05 M phosphate/0.1 M borate buffer at pH 8.09; 8 mM α-cyclodextrin, 1 mM ß-cyclodextrin, and 1 mM γ-cyclodextrin. Peak identification: (1) methanol; (2) 2,4-dichlorophenoxyacetic acid; (3) gibberaalic acid; (4) p-chlorophenoxyacetic; (5) indole-3-butyric acid; (6) 2,4,5-trichlorophenoxyacetic acid; (7) ß-naphthaleneacetic acid; (8) indole-3-propionic acid; (9) α-naphthaleneacetic acid; (10) indole-3-acetic acid [162].

Micellar electrokinetic chromatography (MEKC), a modified capillary electrophoresis, permits the separation of uncharged compounds by electrophoretic technique. However, highly lipophilic compounds, such as corticosteroids, polycyclic aromatic hydrocarbons, fat-soluble vitamins, and polychlorinated biphenyl congeners, could not be resolved by MEKC with sodium dodecylsulfate (SDS) solutions. The addition of cyclodextrin to SDS solution can remarkably improve the resolution of these highly hydrophobic compounds. By using γ-cyclodextrin with SDS in the electrophoretic medium, a mixture of water-soluble and fat-soluble vitamins was successfully separated

simultaneously by MEKC [167]. Recently, a cyclodextrin-modified MEKC (CD-MEKC) system developed by Terabe *et al* [168] has been successfully used to separate highly hydrophobic and closely related compounds including chlorinated benzenes, polychlorinated biphenyl congeners, tetrachlorodibenzo-p-dioxin isomers and polycylic aromatic hydrocarbons.

The use of cyclodextrins as leading electrolyte additives in isotachophoresis has been widely investigated by Jelinek and coworkers [169-177]. The incorporation of cyclodextrin in the background buffer improves the selectivity, thus permiting the efficiently isotachophoretic separation of a wide variety of compounds including penicillins [169], substituted halogenbenzoic acids [160], ephedrine alkaloid enantiomer [171], ketotifen and its polar intermediate enantiomers [172], bile acids [173], structurally related and chiral phenothiazines [174], and the enantiomers of pseudoephedrine, thioridzine, nonpseudoephedrine and hydrothiadene [175].

Fukushi and Hiro [178] studied the effects of α-, ß-, and γ-cyclodextrin on the mobilities of various inorganic anions in capillary isotachophoresis. It was found that the effective mobilities of several anions decreased with increasing cyclodextrin concentration in an ordinary leading electrolyte. By using α-cyclodextrin in the leading electrolyte, nitrite and nitrate ions, cyanate, thiocyanate and selenocyanate ions, chlorate and perchlorate ions were completely separated. Cyclodextrins were also successfully used as leading electrolyte additives in the capillary isotachophoretic separation of positional isomers, such as 2-, 3-, and 4-amino phenols, 1,2-, 1,3-, and 1,4-diaminobenzenes [179], and substituted aromatic sulfonic acids [180]. The incorporation of cyclodextrins within a polyacrylamide gel column can provide a general means of manipulating the selectivity of an electrophoretic separation. As an example of this approach, Guttman [181] reported the electrophoretic separations of dansyl-amino acid enantiomers by incorporoting ß-cyclodextrin in the gel matrix.

1.5.4. Cyclodextrin in gas chromatography

In gas chromatography (GC), both immobilized cyclodextrins and their derivatives, and cyclodextrin polymers have been used as stationary phases.

Several cyclodextrin containing polyurethane resins, cross-linked with different diisocyanates, have been used in GC separations of a series of alcohols, ketones, esters, isomeric xylenes, picolines, and lutidines [182]. The observed elution order for these compounds on α- and ß-cyclodextrin containing resins reflects accurately their expected binding ability to the respective cyclodextrin cavity present in the resins.

Acylated α- and ß-cyclodextrins, such as α-cyclodextrin acetate [183], ß-cyclodextrin acetate [183,184], ß-cyclodextrin -propionate, -butyrate, -valerate [184], and permethylated α- and ß-cyclodextrin [185], have been investigated as stationary phases for GC. For gas-solid chromatography, the stationary phase is prepared by depositing modified cyclodextrin from a dimethylformamide solution onto the support (e.g. Chromosorb W), followed by solvent removal by heating *in vacuo* [186]. For gas-liquid chromatography, the stationary phase is prepared by coating the support with the modified cyclodextrin dissolved in dimethylformamide-ethylene glycol or any other appropriate solvent [187]. A wide variety of compounds including α-olefins, alcohols, aldehydes, aldehyde-esters, diester, isomeric heptadecanoates, unsaturated esters, and saturated fatty acid methyl esters have been separated on these acylated stationary phases [183,184].

Gas chromatographic separations of aliphatic, alicyclic and aromatic hydrocarbons, halogeno derivatives and aliphatic alcohols have also been achieved on α- and ß-cyclodextrin stationary phases [186-189]. The results showed the occurence of inclusion complex formation between the cyclodextrin and the molecules from the gaseous phase.

More recently, the focus of the work involving cyclodextrins in GC has shifted to their utilization as chiral stationary phases. Various modified cyclodextrins have been developed and used as GC chiral stationary phases [190].

Koening *et al.* [191] first reported in 1988 the use of pentylated

cyclodextrins as enantioselective stationary phases for GC. Since that time, the enantiomers of a series of chiral compounds including amino alcohols, amines and amino acids and amino acid esters, O-alkylated glycerols and different lactones, cyanohydrins and carbohydrates, alkyl halides, olefins, ketones diols, triols cyclic acetals, and other hydrocarbons and chiral pharmaceuticals have been separated on the pentylated cyclodextrin GC stationary phases [192-197].

Recently, a series of pentylated cyclodextrin derivatives, 2,6-di-O-pentyl-3-O-trifluoroacetyl (DP-TFA) α-, ß-, and γ-cycylodextrins were developed by Armstrong *et al.* [198, 199] as highly selective chiral stationary phases for capillary gas chromatography. More than 150 pairs of enantiomers were separated by capillary GC with these chiral stationary phases. The enantiomers resolved include chiral alcohols, diols, polyols, amines, amino alcohols, lactones, halohydrocarbons, α-halocarboxylic acid esters, carbohydrates, epoxides, nicotine compounds, pyrans, furans, etc. About 120 of these 150 pairs of enantiomers could be separated on DP-TFA-γ-cyclodextrin stationary phase column, which is the first reported γ-cyclodextrin phase that has a wider resolution spectrum than the ß-cyclodextrin analogue.

Celite coated with α-cyclodextrin has been used as a chiral stationary phase in GC. Using this stationary phase, the separation of enantiomeric mixtures of α-pinene, ß-pinene, limonene and camphene were achieved [200]. Permethylated cyclodextrins were also used in GC chiral separations of racemic alkanediols, substituted carboxylic acid esters, proline methyl ester and heptamethynonane [201], and volatiles belonging to different classes of compounds [202].

A new class of hydrophillic cyclodextrin derivatives, O-(S)-2-hydroxypropyl α-, ß- and γ-cyclodextrins, were recently used as chiral stationary phases for capillary GC [203]. Seventy pairs of enantiomers including chiral alcohols, amines, amino alcohols, epoxides, pyrans, furans, ketones, sugars and bicyclic compounds, etc., were separated on this stationary phase. Figure 1.7 shows the chromatograms for the enantiomeric separation of lactones and bridge-ring compounds on this GC column.

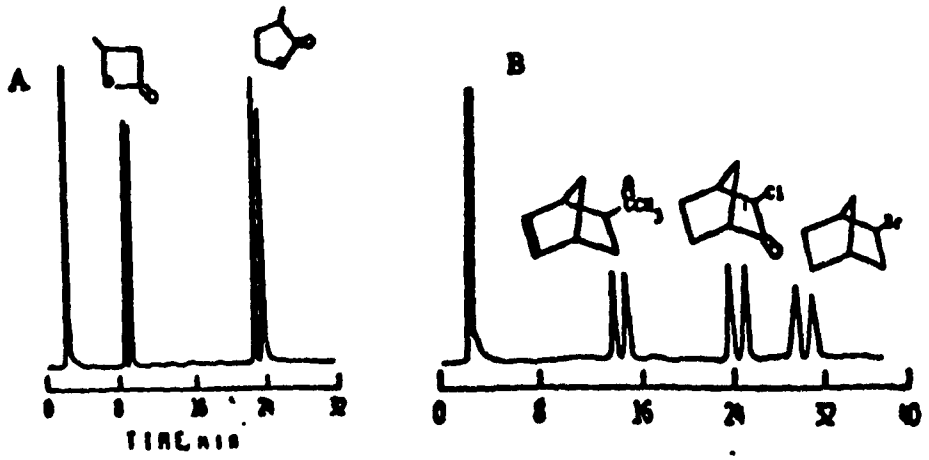


Figure 1.7. Enantiomeric separation of Lactones (A) and bridged-ring compounds on a 9-m fused silica capillary GC column coated with permethyl-O-(S)-2-hydroxy-propyl-derivatizzed ß-cyclodextrin [103].

1.5.5. Cyclodextrin in high performance liquid chromatography

In high performance liquid chromatography (HPLC) the use of cyclodextrins and their derivatives have achieved spectacular success. The uses have been investigated in two different approaches: the use of chemically bonded cyclodextrin-silica as stationary phases and the use of cyclodextrins or highly soluble modified cyclodextrins as the mobile phase additives in a reversed-phase HPLC system.

1.5.5.1. Cyclodextrin bonded stationary phases

In 1965, Solms and Egli [204] first reported the preparation of insoluble cyclodextrin polymers and their selectivity in binding various substances. These first described polymeric cyclodextrin-epichlorohydrin resins, abbreviated ECP, soon became the commonly used LC stationary phases. The separation of various natural products, perfumes, aromatic acids, o- and p-nitrophenols, substituted chlorobenzoic acids, nucleic acids and enantiomeric mandelic acids etc. has been achieved on the cyclodextrin-ECP stationary phase [31]. Several other cyclodextrin containing resins, e.g. cyclodextrin-polyurethane (CDPU) and cyclodextrin-polyvinyl alcohol (CDP), were also developed and used in chromatographic separation of natural amino acids [205, 206], and alkaloids [207]. However, there are some substantial problems associated with the application of cyclodextrin polymeric resins in the HPLC separations. First, the accessibility of the cyclodextrin cavities on the surface and within the interior of the polymer particle is rather different. The entrapment and release of solutes from the mobile phase is a diffusion controlled process, consequently a longer time is needed to reach an equilibrium within the particle than on its surface [35]. Second, liquid chromatography on cyclodextrin polymers can be performed only in aqueous solutions. Third, these soft gels cannot withstand the high pressures used in HPLC. Therefore, the cyclodextrin polymers are rarely used as stationary phases in the HPLC separations.

In recent years, chemically bonded cyclodextrin-silica stationary phases, which are adequate for packings, have been developed [208-211]. The efforts of binding cyclodextrin to a silica matrix by reacting amino modified silica gel with tosylated cyclodextrin have given some reasonable results. The *ortho, meta,* and *para* isomers of several disubstituted benzene derivatives were effectively separated on these stationary phases [210]. However, the use of these nitrogen-containing linkages results in the formation of nitroxides which gives the material a brown color, and renders this material unsuitable for TLC.

In 1985, cyclodextrin bonded stationary phases, which contains no interfering N- or S- linkages, were developed by Armstrong [152] and became

commercially available from Advanced Separation Technologies Inc. (Whippany, New Jersey). These packings consist of cyclodextrin molecules linked to silica gel via a 6-10-atom spacer. Both the linkage and the cyclodextrin are hydrolytically stable under HPLC conditions. The attachment is such that the cyclodextrin molecules remain physically intact. This allows the cyclodextrin column to effect numereous separations by selectively including a wide variety of guest molecules into the cavity.

Cyclodextrin bonded stationary phases have been demonstrated to be particularly adept in resolving structural isomers [212, 213]. The specificity of inclusion complexation allows the successful separation of a series of structural and geometric isomers, such as prostaglandin A_1, A_2, B_1 and B_2, α- and ß-naphthols, o,o'- and p,p'-byphenyls, and the *ortho-*, *meta-* and *para-* isomers of nitrophenol, nitroaniline, xylene, cresol, and amino benzoic acid. In our previous work [214-216], the retention behaviour and separations of 19 chlorinated phenols and 16 chlorinated biphenols were investigated on the ß-cyclodextrin bonded stationary phase. Figure 1.8 shows the gradient resolution of chlorinated phenols. The separation of 15 out of the 19 chlorophenol isomers was achieved within 35 min.

As cyclodextrins are composed of chiral D-glucose units, cyclodextrin complexation provides to be a powerful tool for the separation of other chiral compounds into enantiomers. Cyclodextrin bonded phases have been used for the reversed-phase separation of a wide variety of enantiomers [217, 218]. The details of the application of cyclodextrin stationary phases in chiral separation will be discussed in Chapter 2.

Recently, many modified cyclodextrin stationary phases, which have a broad separation spectrum were developed [219-221]. Some of them have been used for enantiomer separations, even in normal phase HPLC systems [221]. Pawloaska developed a new type of cyclodextrin stationary phase by dynamically coating permethylated ß-cyclodextrin on silica supports [222-224]. This stationary has been used in normal phase HPLC mode for enantiomer separations.

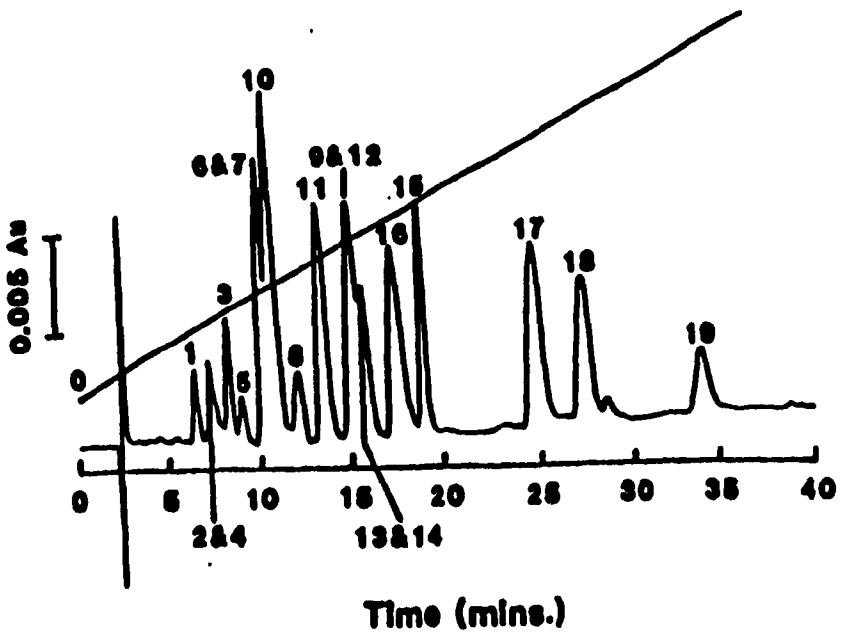


Figure 1.8. Gradient elution separation of chlorophenols (CP) on a ß-cyclodextrin bonded phase column (250 x 4.6 mm). Mobile phase gradient: 27-73% MeOH/H_2O buffer (0.01 M TEAA, pH 4.0); flow rate, 1.0 mL/min; temperature, 50°C. Peak identification: (1) 2-CP; (2) 3-CP; (3) 4-CP; (4) 2,6-diCP; (5) 3,5-diCP; (6) 2,4-diCP; (7) 2,5-diCP; (8) 2,5-diCP; (8) 2,3-diCP; (9) 3,4-diCP; (10) 2,4,6-triCP;(11) 2,3,6-triCP; (12) 2,3,4-triCP; (13) 2,3,5-triCP; (14) 3,4,5,-triCP; (15) 2,4,5-triCP; (16) 2,3,4,5-tetraCP; (17) 2,3,5,6-tetraCP; (18) 2,3,4,6-tetraCP; (19) pentaCP [215].

1.5.5.2. Aqueous cyclodextrin solution as mobile phase

The properties of cyclodextrins, such as: (i) selective and reversible inclusion complexation, (ii) water-solubility, (iii) light-resistant and no

absorption in the full UV range, (iv) stable over a large pH range, promote their use as mobile phase additives in reversed-phase systems. HPLC systems with cyclodextrin present in the mobile phase can realize the separation of various isomers: structural isomers [225], diastereomers [226], as well as enantiomers [227].

Figure 1.9 shows the chromatograms for the separation of o-, m-, and p-isomers of cresol [228], xylene [229], and a mixture of all six isomers of nitrocinnamic acid [230] on the Lichrosorb RP-C18 column with aqueous ß-cyclodextrin solution as mobile phase. Similar results were also observed for o-, m-, and p- isomers of nitrophenol, nitroaniline, fluoronitrobenzene, chloronitrobenzene, iodonitrobenzene, dinitrobenzene [228], mandelic acid derivatives[231] and ethyltoluene [229].

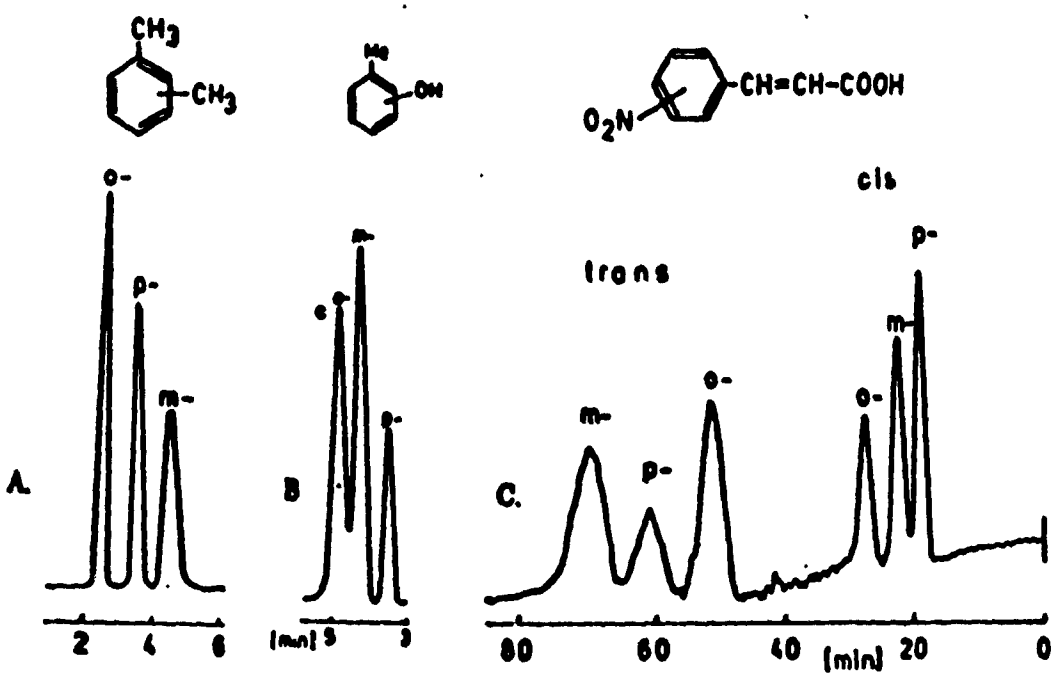


Figure 1.9. Separation of the structural isomers of (A) Xylenees, (B) cresols and (C) cis-tans nitrocinnamic acids on a 10-µm LiChrosorb RP-18 (100 x 4.6 mm i.d.) using aqueous ß-cyclodextrin solution as mobile phase [225].

As illustrated in Figure 1.9, cyclodextrins, especially ß-cyclodextrin, demonstrated high selectivity toward these structural isomers. These highly selective chromatographic separations achieved with a cyclodextrin-containing mobile phase are due to the difference in the stability constants of inclusion complexes in the mobile phase solution and to the difference in the adsorption of these complexes on the stationary phase [225].

Cyclodextrin-containing mobile phases have been successfully used for the enantiomer separations of various chiral compounds including barbiturates, mephenytoin [227], mandelic acid and its derivatives, phenylalanine [232], α-pinene [233], and pseudoephedrine [234].

The cyclodextrin-containing mobile phase has also been used for the separation of specific analytes from complex mixtures. Shimada *et al.* studied the effect of cyclodextrins in the mobile phase on the separation of various compounds including steroids [235, 236], bile acids and their fluorescent derivatives [237, 238], and isomeric estrogens [239]. The separations of these compounds were much improved by the addition of cyclodextrin to the mobile phase.

The use of a cyclodextrin-containing mobile phase not only shows high selectivity and improved separations, but also offers some other significant advantages over the traditional organic solvent or mixed solvent systems [31].

First, since the aqueous cyclodextrin solutions are nontoxic and much less volatile or flammable, the use of cyclodextrin-containing mobile phase is much safer than the currently used organic or mixed solvent mobile phase. Second, the cyclodextrin-containing mobile phase, which is similar to the micellar phases, eliminates most of the solubility problems typically associated with the use of organic solvents, and allows for the simultaneous separation of both nonpolar and polar solutes. Third, the use of cyclodextrin in mobile phase can enhance the chromatographic detection. Cepeda-Saez *et al.* [240] reported that in the LC determination of 5-methoxypsoralen, the addition of 0.01 M ß-cyclodextrin to the MeOH/water (25:75) mobile phase produced a 6-fold increase in the fluorescence signal of 5-methoxypsoralen.

1.6. REFERENCES

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Chapter 2

MOLECULAR CHIRALITY AND CHIRAL SEPARATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.1. MOLECULAR ASYMMETRY AND ENANTIOMERS

In September 1874, Jacobus Henricus van't Hoff, a 22-year-old Dutch scientist, proposed that the four valances of the carbon atom were not planar but were tetrahedral. A young French scientist, Joseph Achille Le Bel, published the same stereochemical hypothesis two months later, in November 1874. Two years later, in 1877, Hermann Kolbe, one of the most distinguished organic chemists of that time, reacted to van't Hoff's publication as follows [1]:

> "Not long ago, I expressed the view that the lack of general education and of thorough training in chemistry was one of the causes of the deterioration of chemical research in Germany.... Will anyone to whom my worries seem exaggerated please read, if he can, a recent memoir by a Herr van't Hoff on 'The Arrangements of Atoms in Space', a document crammed to the hilt with the outpourings of a childish fantasy.... This Dr. J. H. van't Hoff, employed by the Veterinary College at Utrecht, has, so it seems, no taste for accurate chemical research. He finds it more convenient to mount his Pegasus (evidently taken from the stables of the veterinary college) and to announce how, on his bold flight to Mount Parnassus, He saw the atoms arranged in space."

This criticism, although defamatory, made a decisive contribution to the

dissemination of van't Hoff's idea. Within ten years after Kolbe's comments, however, abundant evidence had accumulated to substantiate the "childish fantasy" of van't Hoff, and in 1901 he was named the first recipient of the Nobel Prize for Chemistry. The publications of van't Hoff and Le Bel marked the beginning of a new field: stereochemistry.

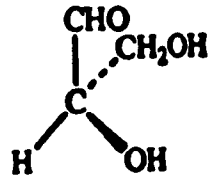
2.1.1. Stereochemical terms and enantiomers

Stereoisomers are compounds which have the same molecular formula, with the same atom-to-atom bonding sequences but with the atoms arranged differently in space. Stereoisomers can be subdivided into two general categories: *enantiomers and diastereomers.*

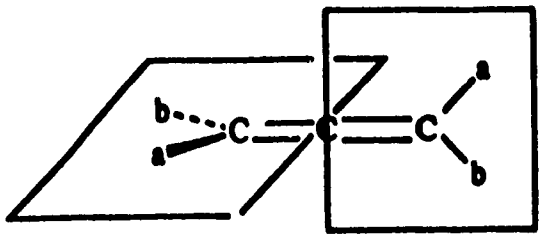
A molecule is called ***chiral*** if it is not superimposable with its mirror image. Molecules (or objects) that are superimposable on their mirror images are ***achiral***. The two different non-superimposable forms of a chiral compound are named ***enantiomers*** or ***optical isomers***. Equimolar mixture of two enantiomers are called ***racemate***. ***Diastereomers*** are stereoisomers whose molecules are not mirror reflection of each other.

Enantiomers exhibit the same chemical composition, constitution and configuration, but have different sterical configurations. A pair of enantiomers will be possible for all molecules that do not possess (i) a plane of symmetry, (ii) a center of symmetry or (iii) any n-fold (n = even number) rotatory alternating axis of symmetry. Figure 2.1 illustrates some typical examples of chiral molecules [2]. As can be seen from these examples, chirality in a molecule may be caused by one of the following reasons: (1) the presence of a chiral atom, e.g. an atom with four different groups attached to it; (2) twisted structures, such as cumulenes and spiranes; (3) hindered rotation, such as

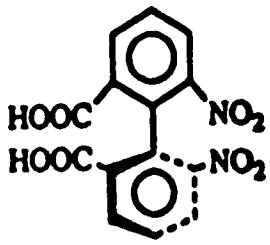
1. Asymmetric Center



2. Twisted Structure



3. Hindered Rotation



4. Molecular Overcrowding

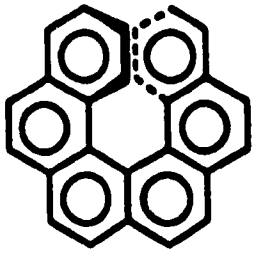


Figure 2.1. Chiral molecules.

biaryls and ansa compounds; and (4) molecular overcrowding, such as polycyclic aromatics.

Chiral atoms can be any tetrahedral atom with four different groups attached to it [3]. Figure 2.2. lists some examples of compounds whose molecules contain chiral atoms other than carbon. Chirality is not a unique feature of the asymmetric organic compounds. In fact, a few purely inorganic molecular species also exhibit chirality [4].

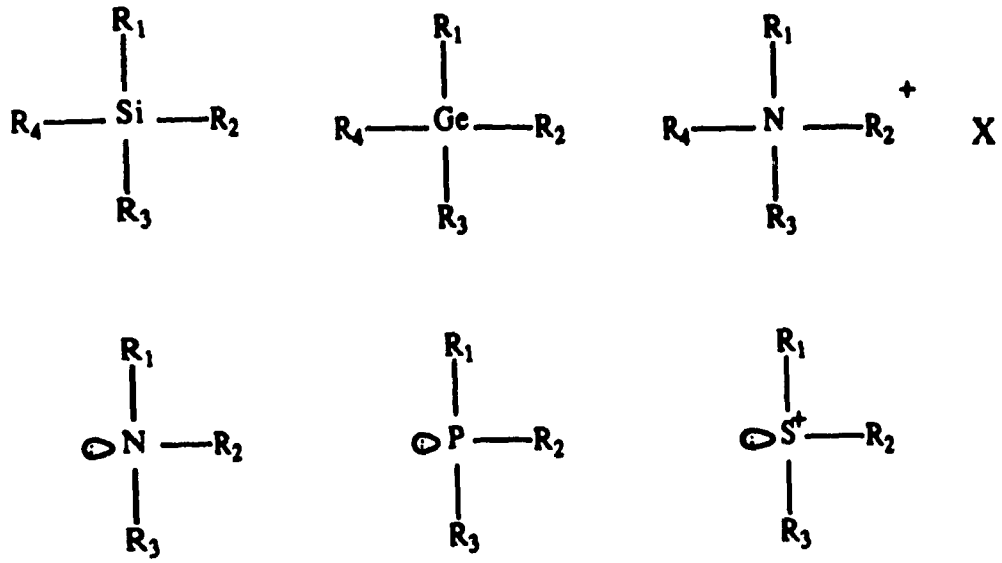


Figure 2.2. Chiral compounds with chiral centers other than carbon atom.

In the literature, three different nomenclature systems can be found for enantiomers. The older designations D and L are assigned by deriving the structure of the enantiomer from D- and L- glyceric aldehyde. The (+) and (-) designations indicate the direction of rotation of plain polarized light by the respective enantiomer. The R and S system, devised by Cahn, Ingold and Prelog, stands for the absolute configuration of the substituents on the asymmetric carbon atom.

2.1.2. Properties of enantiomers

Unlike the structural isomers, enantiomers have the same chemical and

physical properties in an achiral environment. They have the same melting and boiling points, the same indexes of reflection, the same solubilities in common solvents, the same infrared spectra, and the same rates of reaction with achiral reagents.

Enantiomers show different behaviour only when they are in a chiral environment or interact with other chiral substances. Enantiomers show different solubilities in chiral solvents. They also show different rates of reaction with reagents consisting of a single enantiomer or an excess of a single enantiomer.

Enantiomers differ in their behaviour toward plane-polarized light. When a beam of plane-polarized light passes through an enantiomer, the vibration plane of the plane-polarized light rotates. This rotation is due to the different propagation rates of the R and L circularly polarized components of the plane-polarized light in a optically active medium. One of the enantiomers rotates the beam in a counterclockwise direction, and is defined as the levorotatory or *l*-enantiomer (or (-)-enantiomer). The other enantiomer rotates the plane of the plane-polarized light equal amounts but in the opposite direction (clockwise), and is defined as the dextrorotatory or d-enantiomer (or (+)-enantiomer).

2.1.3. The differences of enantiomers in their pharmaceutical and biological activities

At first sight, fauna (human beings and animals) show a high degree of symmetry, but on the molecular level, asymmetry dominates. The human body is a highly stereospecific environment (we have D-sugars and L-amino acids). It is clear that, within the body, the enantiomers exist in a highly chiral environment where their release, absorption, transportion, action, degradation and elimination may involve interaction with enzymes, cell surfaces and so on,

all of which are asymmetric at the molecular level. In this highly stereospecific environment the behaviour of enantiomers can be very different.

Over the last decade, many studies have indicated that the biological or pharmacological activities and effectiveness of the two enantiomeric forms of a chiral drug can be significantly different. Often, only one of the enantiomers is pharmacologically active, while the other may at best be less active or inactive, or even toxic [5]. For example, the S-enantiomer of propranolol is about 100 times more potent than the R-enantiomer in blocking the inotropic and chronotropic response to isoproterenol [6]. Bufenadrine was developed as an antimotion sickness drug, but was not put on the market because chronic toxicity studies in rats showed liver toxicity. Subsequent toxicity studies with the optical isomers indicated that the (-) enantiomer was mainly responsible for the liver toxicity [5, 7]. Table 2.1 shows some other examples for the different activities of the two enantiomers of one drug [8].

The two enantiomers of a chiral drug may also show differences in metabolism and pharmacokinetics. In acenocoumarol, for example, the total body clearances for (+)-enantiomer and (-)-enantiomer are 35 mL/min and 496 mL/min, respectively [9]. The ratio of the more rapidly cleared enantiomer to the less rapidly cleared enantiomer is as large as 14.0. Also, drug enantiomers can be metabolized differently by the different pathways of biotransformation. In warfarin, for example, the R-(+)-enantiomer is biotransformed mainly by oxidation to 6- and 8-hydroxywarfarin, whereas the S(-)-enantiomer is eliminated mainly by oxidation to 7-hydroxywarfarin [10].

Table 2.1. The differences of the enantiomers of a chiral drug in pharmacological activities

Pharmaceuticals	Effects
Estone	(+) estrogenic hormone (-) inactive
Asparagine	(R) sweet (S) bitter
DOPA	(L) treatment of Parkisons disease
Penicillamine	(D) antiarthritic (L) extremely toxic
Thyroxine	(S) thyroid gland hormone (R) antihyperchlolesterinic
Ethambutol	(SS) tuberculostatic (RR) blindness
Thalidomide	(R) sleep inducing (S) teratogenic

2.2. ENANTIOMER SEPARATIONS

2.2.1. The importance of enantiomer separations

The separation of enantiomers has long been a challenging field to analytical chemists. In recent years, there was a rapidly increasing interest in this field. This interest is due to the fact that many of the chemical products closely related to human health, such as pharmaceuticals, food additives and agrochemicals, are chiral compounds and have been marketed as racemic mixtures. The enantiomeric molecules often differ in potency, toxicity, pharmacological action, metabolism, and taste.

It has been reported that about 57% of the active drugs prescribed in the United States contain at least one chiral atom, and about 45% of these chiral drugs are marketed as racemic mixtures [11, 12]. The examples presented in Section 2.1.3 have clearly demonstrated that the biological and pharmacological activities of the chiral molecules depend largely on their configurations. For most chiral drugs, further investigations are necessary to understand the differences in the activity, toxicity and metabolic pathway of the two enantiomers. Therefore, the ability to rapidly and accurately separate and determine the enantiomeric composition of chiral drugs is becoming increasingly important in drug development, regulation and clinical applications.

Enantiomeric separation can also make important contributions to food science. Many food additives (e.g. flavour and fragrance components) are chiral compounds. A variety of receptors on the human tongue and nasal membranes are stereoselective for certain compounds. R-carvone, for example, smells like mint while the S-carvone smells like caraway. Another example is asparagine. The R-asparagine tastes sweet while the S-asparagine tastes bitter [8]. The ability to accurately resolve the enantiomers of these chiral compounds may allow one to more accurately evaluate and produce the desired flavours and fragrances.

Chiral separation and optical purity determination have even been used for the dating of archaeological materials [13]. It is well known that only L-

amino acids are usually found in the protein of living organisms, but in the dead body, these L-amino acids undergo slow racemisation, producing the D-amino acids. Over long periods of geological time they form equilibrium mixture of D- and L-enantiomers.

L-amino acid ⇌ D-amino acid

Thus, by determining the extent of racemisation in a fossil material, its age can be estimated.

2.2.2. Enantiomer separation methods

Enantiomers have identical solubilities in ordinary solvents, and they have identical melting and boiling points. Consequently, the conventional methods for separating organic compounds, such as crystallization, distillation, extraction and ordinary chromatographic methods fail when applied to a racemate.

In 1848, Louis Pasteur [14], using tweezers, separated the non-identical crystals of the enantiomers of sodium ammonium tartrate under a microscope. Unfortunately, few chiral compounds give separate crystals that are visibly chiral like the crystals of the sodium ammonium salt of tartaric acid. Pasteur's manual method, therefore, is not generally applicable. Ten years later, Pasteur discovered a method for resolving racemic paratartaric acid by using bacteria to selectively destroy one of the enantiomers. Pasteur's new method, later qualified, modified and generalized by others, became applicable to the separation of a number of other racemates [2].

After Pasteur's pioneering work, many different methods were investigated. Several naturally-occurring chiral materials, such as wool, silk, quartz, starch and cellulose, had been utilized for resolving the racemates. The most useful procedure for enantiomer separation was based on allowing a racemate to react with a single enantiomer of some other chiral compounds.

This reaction changes a racemate into a mixtures of diastereomers. The diastereomers, because they have different physical and chemical properties, can be separated by conventional means. This is the most fundamental approach used for the enantiomeric separations today.

In the 1930s, column chromatography with lactose as the adsorbent was successfully employed by Henderson and Rule [15] for the separation of a racemic camphor derivative. In 1951, Kotake *et al.* [16] resolved the enantiomers of amino acids by paper chromatography, and attributed the resolution to the chirality of the cellulose. In 1952, Dalgliesh [17] resolved the enantiomers of amino acids by paper chromatography and postulated a three-point interaction model between the chiral solute and the chiral stationary phase. In the 1960s, the direct separation of enantiomers by GC was achieved by Gil-Av *et al.* [18], using chiral stationary phases. Also, at about the same time GC and liquid chromatography with achiral stationary phases were often used for the resolution of enantiomers after derivatization with chiral reagents.

The increasing popularity of HPLC and the advances in HPLC packing materials in the 1970s encouraged many researchers to develop chiral stationary phases for direct HPLC separation of the racemic mixtures. Then, in the 1980s, chiral liquid chromatography witnessed an explosive growth in popularity. Today, more than 50 different chiral stationary phases are commercially available for direct chiral liquid chromatographic separations.

With rapid progress in chiral separation, the modern analyst has several different approaches that can be employed for the enantiomer separations [19]:

(i) derivatization using chiral reagents then separation by HPLC, GC, TLC, or capillary electrophoresis (CE);
(ii) chiral stationary phases for HPLC, GC, and TLC;
(iii) chiral mobile phases for HPLC, TLC and CE.

Compared to HPLC, GC has the disadvantage that the higher operating temperatures required may lead to racemization of both the chiral stationary phase and the enantiomers to be separated. Compared to TLC, HPLC provides greater sensitivity and better reproducibility [20]. Therefore, HPLC is the method of choice. In fact, HPLC has predominated in the research area of

enantiomeric separations. A wide variety of structural types of enantiomers have been separated by liquid chromatographic methods.

2.3. CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chiral separations by HPLC are currently enjoying a widespread popularity. Several books [21-23] and review articles [24-27] have been devoted to presenting the current state of the art in enantiomeric separations by the HPLC method. In HPLC, enantiomer separation can generally be achieved in three different ways:

(1) direct separation of enantiomers with chiral stationary phases (CSP);

(2) chiral solvents or chiral additives to the mobile phase induces temporary diastereomers between the enantiomers and the chiral mobile phase additives;

(3) precolumn derivatization with chiral reagents produces diastereomers which can be separated by non-chiral stationary phases.

In order to use the third approach successfully, the reagent must be readily available in a chemically and optically pure form and must react at the same rate with both enantiomers. Furthermore, racemization, decomposition and side reaction must not occur during the derivatization. These prerequisites limit the usefulness of this approach. In this section, the discussion will be limited to the first and second approaches.

2.3.1. Theory of chiral recognition on CSP --- three-point rule

As mentioned before, direct separation of enantiomers requires a chiral environment. In order for a CSP to work, it must be able to form transient

diastereomeric complexes with at least one of the two enantiomers when the enantiomers pass over it. The free energy of formation for these diastereomeric complexes must be different. Figure 2.3 shows the diagram of a chiral resolution process on an HPLC CSP. Before injection, the free energies of the two enantiomers are identical. The same is true after elution from the column. On the column, however, a difference in the free energies ($\Delta(\Delta G)$) is produced through the formation of the transient diastereomeric complexes between the enantiomer and the chiral selector bonded to the CSP. The resolution of the enantiomers depends on the value of $\Delta(\Delta G)$. Pirkle and Pochapsky [24] derived an expression for the relationship between the separation factor (α_{SR}) and the difference in free energy ($\Delta(\Delta G)$) as the following:

$$\Delta(\Delta G) = -RT \ln \alpha_{SR}$$

where $\alpha_{SR} = k_S/k_R$. k_S and k_R are the capacity factors of S- and R-enantiomer, respectively.

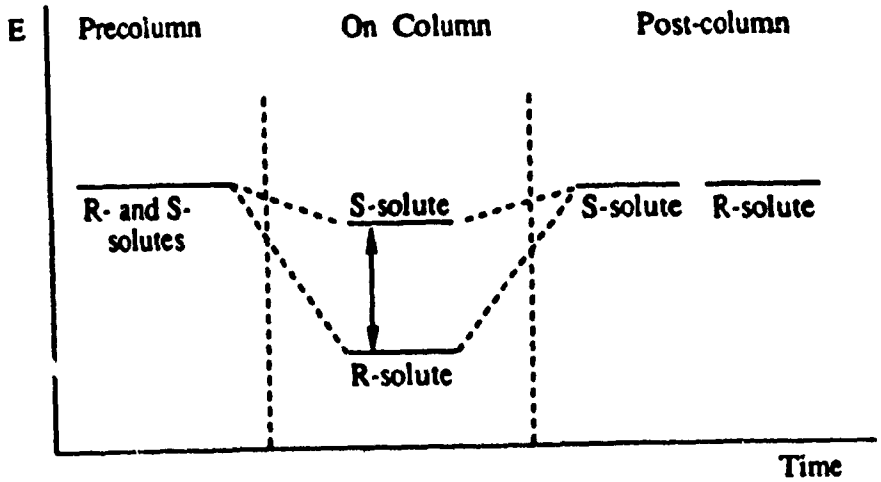


Figure 2.3. General chiral separation mechanism on a chiral stationary phase.

How does chiral recognition occur? This has long been a subject investigated by many researchers. In 1933, Easson and Stedman [28] first postulated a 'three-point' interaction model when discussing the interaction of racemic drugs with receptors as well as substrate interactions. This 'three-point' rule is based on the fact that the determination of the configuration of a chiral carbon involves at least three of the four bonds attached to that chiral centre. In 1952, Dalgliesh [17] first applied the 'three-point' model for the explanation of the resolution of amino acids by cellulose paper chromatography. Pirkle and Pochapsky restated the 'three-point' rule as the following:

> "Chiral recognition requires a minimum of three simultaneous interactions between the CSP and at least one of the enantiomers, with at least one of these interactions being stereochemically dependent."

Figure 2.4 illustrates the generalized representation of the 'three-point' rule. In this case, enantiomer (a) interacts with the CSP at three different sites: A-W, E-X and D-Y. Whereas its mirror reflection, enantiomer (b), lacks the A-W interaction. If the A-W interaction is attractive, the diastereomeric complex of enantiomer (a)-CSP will be more stable than the complex of enantiomer (b)-CSP, thus enantiomer (a) will be retained on the column longer than enantiomer (b). If the A-W interaction is repulsive, the situation will be reversed and enantiomer (a) will be eluted first. If A-W interacts minimally or not at all, no separation is expected.

It should be pointed out that the 'three-point' interaction rule does not mean that chiral resolution requires all three points of attractive or repulsive interaction. It means that at least three of the stereochemical elements of the solute and the CSP must be involved in the chiral recognition process [25].

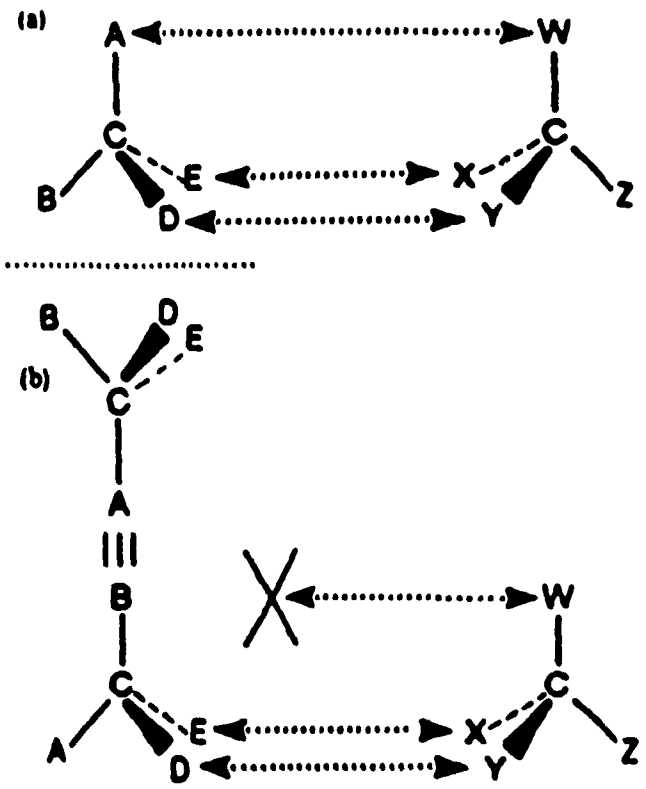


Figure 2.4. "Three-points" interaction rule.

2.3.2. Chiral stationary phases

According to their chiral recognition processes, the current commercially available chiral stationary phases (CSPs) can be classified into five categories:

(1). Ligand-exchange CSPs.
(2). Donor-acceptor CSPs.
(3). Protein type CSPs.
(4). Helical polymer CSPs.
(5). CSPs with chiral cavities.

2.3.2.1. Ligand-exchange chiral stationary phases

Ligand-exchange CSPs were first developed by Rogozhin and Davankov [29] in 1968. They prepared various ligand-exchange stationary phases from chloromethylated polystyrene containing optically active bifunctional and trifunctional α-amino acids. After treatment with transition metal ions, such as Cu(II), Ni(II), Zn(II) and Cd(II), these chiral ligand exchange resins were shown to readily resolve the racemates of amino acids. The separation is believed to be caused by the formation of diastereomeric chelate complexes. Gubitz and co-workers [30,31] used the same principle and bonded chiral ligand of amino acid, L-proline or L-valine, to silica gel via a 3-glycidoxypropyl spacer. After loading with copper ion, these amino acid bonded stationary phases are ready for enantioselective formation with other amino acids. Figure 2.5 illustrates the proposed chiral recognition mechanism.This bonded silica stationary phase improved the column efficiency, thus achieving good resolution for the racemic amino acids.

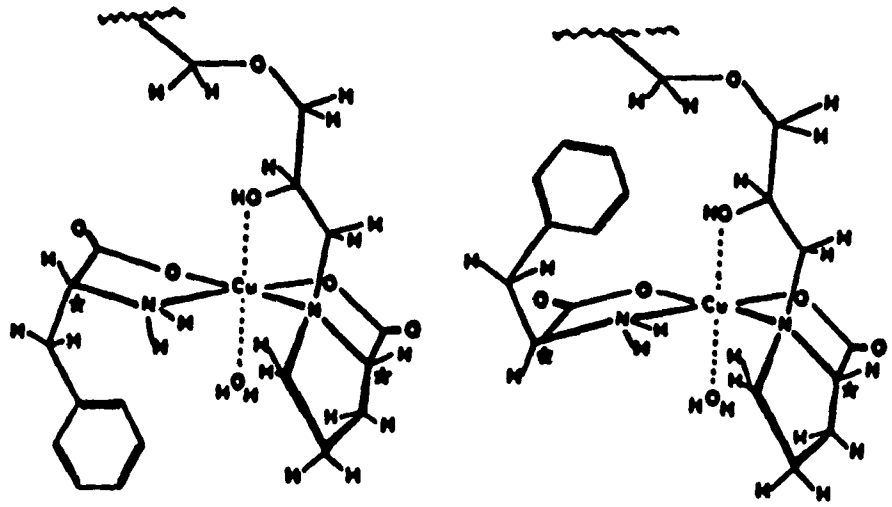


Figure 2.5. Chiral recognition of D- (left) and L-phenylalanine methyl ester (right) with copper-loaded L-proline bonded stationary phase.

In 1987, Shieh *et al.* [32] developed an L-proline a bonded phase diluted with C_{18} alkyl groups. Such a stationary phase has been applied to the resolution of amino alcohol enantiomers.

Ligand-exchange CSPs are now commercially available from several companies. They are mainly used for the enantiomeric separation of those chiral compounds which have two or more polar functional groups with the correct spacing, such as amino acids and amino acid-like substances.

Ligand-exchange CSPs are normally used with an aqueous mobile phase containing certain amounts of transient metal ions to prevent a loss of copper from the stationary phase. Kurganov *et al.* [33] demonstrated that nonaqueous mobile phase could also be used for ligand-exchange CSPs but, so far, the non-aqueous mobile phases has only been applied to the separation of the enantiomers of organometallic complexes.

2.3.2.2. Donor-acceptor chiral stationary phases

Donor-acceptor type CSPs, also known as Pirkle type or 'brush type', comprise the largest section of the commercially available HPLC CSPs. The first commercially available donor-acceptor CSP was developed by Pirkle *et al.* [34] by ionically binding N-(3,5-dinitrobenzoyl)phenylglycine to aminopropyl-silica. This phase was designed to operate using hydrogen-bonding, π donor-acceptor, dipole-stacking and steric interactions between the CSP and the solute. Since this pioneering work, a number of other donor acceptor CSPs have been developed using the same principle. Figure 2.6 shows the structures of some commercially available donor-acceptor phases.

Donor-acceptor CSPs have been successfully used to separate the enantiomers of a wide variety of chiral compounds [35], including alcohols, thiols, amines, amino acids, amino alcohols, cyclic imidides, benodiazepenones, carboxylic acids, phthalides, sulphoxides, phosphine oxides, binaphthols, lactams and succinimides. Donor-acceptor CSPs have perhaps a wider applicability than any other class of CSPs. However, they have also some limitations. They often require relatively lipophilic solutes. Strongly cationic molecules, such as amines, and strongly anionic molecules, such as carboxylic

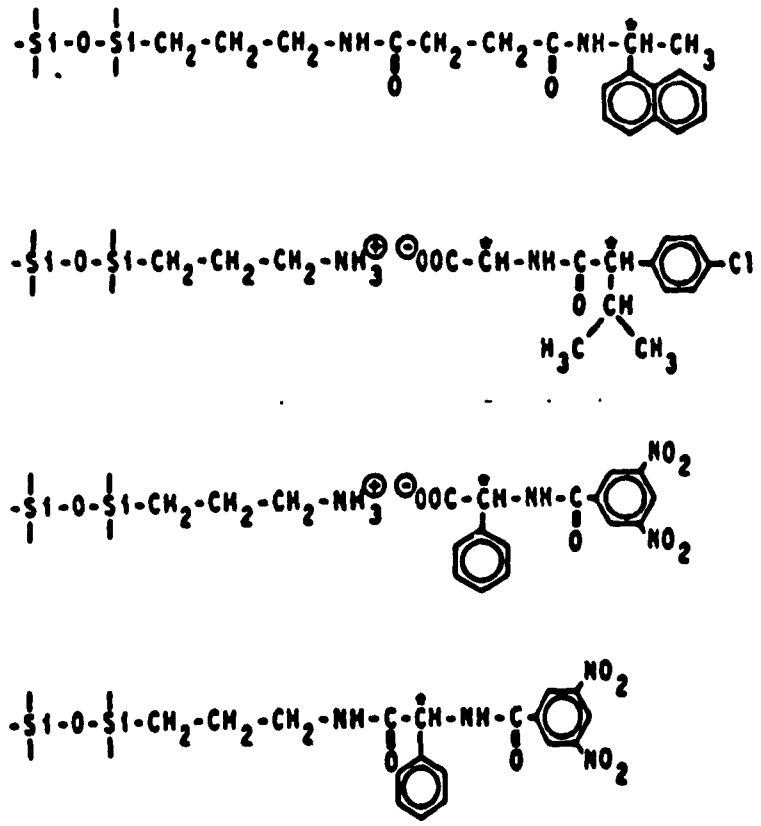


Figure 2.6. Structures of donor-acceptor chiral stationary phases.

acids, are not easily resolved on these CSPs. These solutes usually need to be derivatized to amides or esters.

Donor-acceptor CSPs are commonly used with a non-polar mobile phase composed of hexane and an alcoholic polar modifier, such as propanol.

2.3.2.3. Protein type chiral stationary phases

Another type of widely used CSPs is protein-bonded phases. So far, three different protein-bonded silica CSPs have been developed. They are bovine

serum albumin (BSA) columns [36,37], α_1-acid glycoprotein (AGP) columns [38] and ovomucoid columns [39].

Protein CSPs are excellently suited for the separation of racemic pharmaceuticals, and often show very high separation factors. The enantiomers of a wide variety of pharmaceuticals, such as barbiturates [40], hydantoins [41], benzothiadiazines [41], benzodiazepinones [42], anionic drugs (ibuprofen, ketoprofen, and naproxen) and cationic compounds. have been separated on these protein bonded phases.

The chiral recognition process of the protein CSPs appears to involve a combination of hydrogen-bonding, hydrophobic interactions, ion-pairing and charge-transfer interactions.

The protein CSPs are normally used in reversed-phase mode with a mobile phase containing a phosphate buffer and an organic modifier. The retention and resolution depend largely on the chromatographic conditions, such as mobile phase composition, pH, ionic strength, and column temperature. The recommended conditions are: pH, 5-9; ionic strength, 0-0.5 M; organic modifiers up to 5% propanol; and temperature of less than 35°C [27].

2.3.2.4. Helical polymer chiral stationary phases

Polymers with one-handed helical structures are able to separate enantiomers by steric effects. Cellulose, which has a helical structure, is an obvious choice as a candidate for CSPs. However, native cellulose has several drawbacks, such as poor mechanical properties, high polarity and porous structure, giving unfavourable chromatographic behaviour. In 1973, Hesse and Hagel [43] prepared a microcrystalline cellulose (cellulose triacetate derivative) and successfully used it for enantiomeric separations. In 1984, Ichida *et al.* [44] developed a series of cellulose CSPs by coating the peracrylated cellulose onto diphenyl-silanized macroporous silica gel. These cellulose phases provide a fairly durable and noncompressible chromatographic medium with improved chromatographic behaviour.

Currently, nine different cellulose stationary phases are commercially

available. They are the microcrystalline cellulose triacetate (CSP1) and eight cellulose derivatives coated on silica gel, namely, triacetate (CSP2), tribenzoate (CSP3), trisphenyl carbamate (CSP4), tricinnamate (CSP5), tris(3,5-dimethylphenyl) carbamate (CSP6), tris(4-chlorophenyl) carbamate (CSP7), tris(4-methylphenyl) carbamate (CSP8) and tris-4-methylbenzoate (CSP9). These cellulosic CSPs have proved to be powerful tools for the enantiomeric separation of those chiral compounds which have one or more aromatic ring(s) or polar π-bonded groups such as carbonyl, sulfinyl, or nitro groups. The separated chiral compounds include axially and planar dissymmetric compounds (such as biaryls, cyclophanes, cumulene and spiranes), sterically hindered amides and related compounds, organometallic complexes, compounds possessing chiral sulphur or phosphorous atoms, nitro and cyano compounds, ketones, amines, alcohols, carboxylic acids and ethers [45].

Cellulose CSPs are usually used with a non-polar mobile phase composed of hexane modified with alcohols.

The successful use of cellulose CSPs has inspired researchers to develop a number of synthetic chiral polymers. The typical examples of this type of optically active polymers are poly(triphenylmethyl methacrylate) (PTrMA) [46] and poly(diphenyl-2-pyridylmethyl methacrylate) (PD2PyMA) [47]. The chiral compounds resolved on these CSPs are axially and planar dissymmetric compounds, metal-containing compounds, alcohols, amines, esters, ketones and amides [49].

2.3.2.5. Cavity chiral stationary phases

The significant advance in chiral liquid chromatography was the commercial introduction of cyclodextrin-bonded stationary phases. As mentioned in Chapter 1, cyclodextrins have the ability to form inclusion complexes with a wide variety of compounds. In many cases, the inclusion complexation is enantioselective. In 1984, Armstrong and Demond [49] developed the cyclodextrin-bonded stationary phases by bonding cyclodextrins to silica gel. Currently, α-, ß-, and γ-cyclodextrin columns as well as the acetylated versions of these columns are commercially available from Astec.

Cyclodextrin stationary phases show several advantages over other CSPs. First, they are designed to operate in the reversed-phase mode. Second, they are chemically and physically robust. Third, there are several different cavity sizes, so they can be used to separate a variety of different sized enantiomers. The enantiomers separated on cyclodextrin CSPs include axially and planar dissymmetric compounds, amines, amino acids and their derivatives, metallocenes, barbiturates, and nornicotines [50].

Crown ethers immobilized on silica gel are another type of cavity CSPs. So far, three different types of crown ether CSPs have been developed using different methods of immobilization, e.g. polymerization [51], bonded to silica [52, 53], and physical adsorption onto the silica [54, 55]. The successful enantiomer separation by chiral crown ether stationary phases has been reported by several research groups [56-59]. The chiral compounds resolved on crown ether CSPs are racemic amines, amino acids and amino acid derivatives. The chiral recognition mechanism is based on the formation of a diastereomeric complex between the chiral crown cavity and the solute (see Figure 2.7) [58].

Crown ether CSPs usually operate in the reversed-phase mode. Aqueous methanol solution containing an organic modifier is often used as mobile phase.

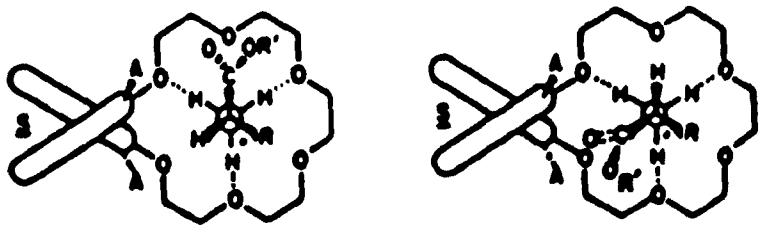


Figure 2.7. Diastereomeric complexes between 3,3'-substituted crown ether and amino ester [58].

2.3.3. Chiral mobile phases

The separation of enantiomers on chiral stationary phases involves the formation of reversible diastereomeric complexes. The same effect can sometimes be achieved by adding optically active reagents to the mobile phase and using achiral stationary phases. Excluding cyclodextrins, which have been discussed in Chapter 1, several other types of optically active reagents have also been successfully used as chiral mobile phase additives.

A mobile phase additive consisting of a complex of a chiral ligand with a metal ion has been successfully used for the enantiomeric separation of amino acids. Chiral recognition is based on a ligand-exchange process, and the diastereomeric ternary complexation is the driving force for the enantioselectivity in the chromatographic process. The chiral ligands used in this technique should have two or more functional groups for chelate bonding in the vicinity of its chiral center, have a bulky residue to induce repulsion, and be optically pure. L-Proline, L-arginine and L-phenylalanine are the most commonly used chiral ligands. The metal ion in the chiral complex should be transition metal ions, such as Cu(II), Zn(II), Ni(II), Co(II), Fe(II), Fe(III), and Cd(II). Like the ligand-exchange CSPs, this technique can be used for the enantiomer separation of only those compounds which have two or more chelating functional groups, such as amino acids, hydroxy carboxylic acids, ß-amino alcohols, and so on [60].

Optically active ion-pair reagents have also been used as chiral mobile phase additives for the enantiomer separations. The resolution is based on the formation of diastereomeric ion-pair complexes with different stabilities and distribution properties between the mobile and the stationary phases. This approach has been successfully used for the separation of racemic ß-aminoalcohols [61, 62], carboxylic and sulphonic acids [63] and organometallic compounds [64]. The ion-pair reagents are generally used with non-polar mobile phases to promote a high degree of ion-pair formation.

Some crown ethers (mainly 18-crown-6 ether bearing 1,1'-binaphthyl moiety) have been successfully used as chiral mobile phase additives in normal phase liquid chromatography. As an example of this approach, Figure 2.8 shows the enantiomeric separation of phenylglycine methyl ester on a silica gel

column with crown ether as chiral the mobile phase additive [65].

Chiral separation with a chiral mobile phase offers the following advantages: (1) less expensive and more common column packings can be used; (2) there is a wider choice of optically active reagents than chiral stationary phases; and (3) it can also be used for preparative isolation.

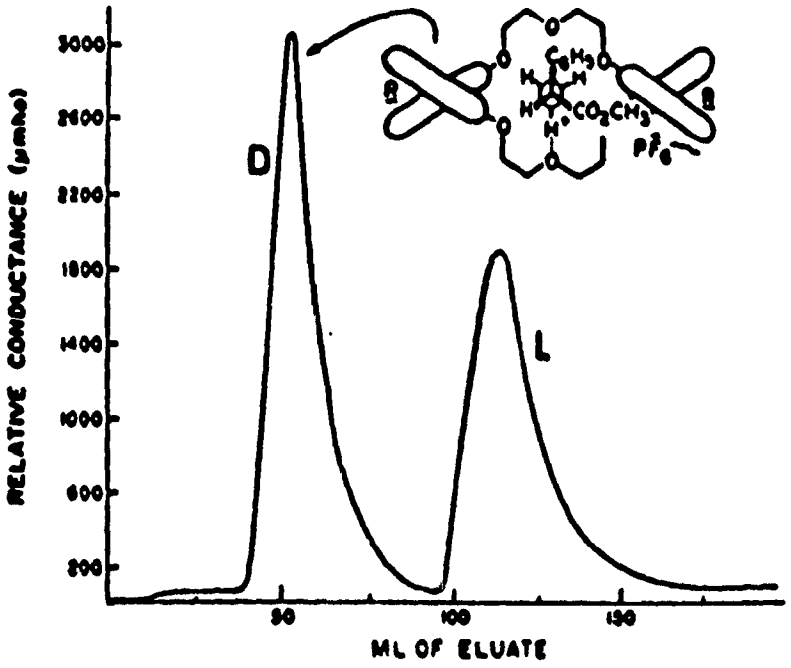


Figure 2.8. Enantiomer separation of racemic phenylglycine methyl ester using crown ether as a mobile phase additive in normal-phase HPLC. Stationary phase, silica gel treated with aqueous $NaPF_6$ solution, mobile phase, 37.8 mM crown ether in $CHCl_3$ solvent [65].

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Chapter 3

LIQUID CHROMATOGRAPHIC SEPARATIONS OF PHENOTHIAZINE AND ITS STRUCTURALLY-RELATED COMPOUNDS ON A ß-CYCLODEXTRIN-BONDED PHASE COLUMN

3.1. INTRODUCTION

Phenothiazine and its derivatives are an important group of drugs possessing antiemetic, antipsychotic, sedative, antipruritic, antidyskinetic, analgesic and antihistaminic properties [1-3]. More than 100 phenothiazine derivative drugs have been synthesized and pharmacologically tested during the past few decades [1,2]. Separation and quantification of these compounds and their metabolites is necessary in clinical studies and in analytical toxicology to diagnose possible intoxication.

Although these compounds are closely related chemically, there is no uniformity in their separation and quantification. A number of investigators [4-7] have described thin-layer chromatography (TLC) systems that are useful in the separation and identification of phenothiazine and its structurally-related compounds. A TLC system applicable to the separation of 40 phenothiazines and their sulphoxides was developed by Kofoed *et al.* [4]. Separation and detection of some phenothiazine drugs using gas chromatography has also been described [8, 9]. A gas chromatography-mass

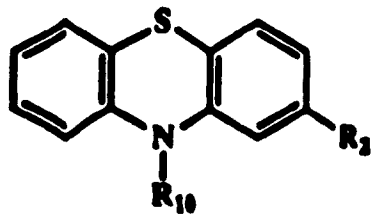
spectrometric (GC-MS) technique for the identification of phenothiazines and analogous neuroleptics was reported by Maurer and Pfleger [10]. Jelinek and Dohnal [11] employed an isotachophoresic system to separate 11 phenothiazine and structurally-related compounds. Liquid chromatography (LC) has also been applied to the separation and detection of some phenothiazine derivatives, such as promethazine [12], thioridazine [13], and fluphenazine and its esters [14]. However, some of the phenothiazine derivatives are very difficult to separate by LC methods based on adsorption, ion-exchange or reversed-phase partition processes.

As presented in Chapter 1, cyclodextrin-bonded stationary phases for liquid chromatography have been demonstrated to be particularly adept in many difficult separations [15-18], such as the separation of enantiomers, diastereomers, and positional, geometric and structural isomers. The basic property of cyclodextrin bonded stationary phases that allows them to accomplish many difficult separations is their ability to form selective inclusion complexes with a wide variety of guest molecules. The inclusion complexation depends on the structure of the solutes considered as a whole rather than as a function of specific functional groups. Therefore, cyclodextrin stationary phases may substantially improve the separation of phenothiazine and its structurally-related drugs.

In our previous studies [19 20], the separation of all the chlorophenols (19 isomers) and 15 chlorobiphenols has been achieved on a single ß-cyclodextrin column [19,20]. This chapter reports the results for the study of the liquid chromatographic retention behaviour and separations of 16 phenothiazine and its structurally-related drugs (Table 3.1) on a ß-cyclodextrin bonded phase column. These structurally related compounds can be used as model solutes for the study of various separation mechanisms and for the testing the capabilities of a proposed analytical system. The retention

Table 3.1.

Structures of the compounds investigated.



No.	Name	R_{10}	R_2
1	Propiomazine	$-CH(CH_3)CH_2N(CH_3)_2$	$-COC_2H_5$
2	Ethopropazine	$-CH_2CH(CH_3)N(C_2H_5)_2$	-H
3	Promethazine	$-CH_2CH(CH_3)N(CH_3)_2$	-H
4	2-Acetylphenothiazine	-H	$-COCH_3$
5	Acetopromazine	$-(CH_2)_3N(CH_3)_2$	$-COCH_3$
6	Triflupromazine	$-(CH_2)_3N(CH_3)_2$	$-CF_3$
7	2-(trifluoromethyl)phenothiazine	-H	$-CF_3$
8	Promazine	$-(CH_2)_3N(CH_3)_2$	-H
9	Phenothiazine	-H	-H
10	Trimeprazine	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	-H
11	2-methoxylphenothiazine	-H	$-OCH_3$
12	Chlorpromazine	$-(CH_2)_3N(CH_3)_2$	-Cl
13	Trifluoperazine	$-(CH_2)_2$-N⟨piperazine⟩N-CH_3	$-CF_3$
14	Perphenazine	$-(CH_2)_3$-N⟨piperazine⟩N-CH_2CH_2OH	-Cl
15	Thioridazine	$-(CH_2)_2$-⟨piperidine⟩-N-CH_3	$-SCH_3$
16	Prochlorperazine	$-(CH_2)_3$-N⟨piperazine⟩N-CH_3	-Cl

behaviour on the ß-cyclodextrin stationary phase is investigated with respect to methanol and triethylammonium acetate (TEAA) buffer concentration in the mobile phase, pH, and column temperature. The liquid chromatographic separations of these 16 compounds are carried out in both isocratic elution and gradient elution modes.

3.2. EXPERIMENTAL

3.2.1. Apparatus.

Chromatography was performed using a liquid chromatographic system that consisted of two Model 590 pumps (Waters Associates, Milford, MA, USA), a Model 660 solvent programmer (Waters), a Model 7125 injector equipped with a 10-µl loop (Rheodyne, Cotati, CA, USA), and a Model 440 UV detector (Waters Associates). The chromatograms were recorded on a Model SE120 strip chart recorder (Goerz Electro, Austria). The column temperature was controlled by a HETO 623 water bath (Bach-Simpson Limited, London, Ontario, Canada).

A Cyclobond I column (250 x 4.6 mm) was purchased from Advanced Separation Technologies (Whippany, NJ, USA). The Cyclobond I column contains ß-cyclodextrin molecules chemically bonded to spherical silica gel When not in use, the column was stored in 100% methanol.

3.2.2. Chemicals

2-Acetylphenothiazine, 2-(trifluoromethyl)phenothiazine and 2-methoxyphenothiazine were obtained from Chemical Dynamics Co. (South Plainfield, NJ, USA). All other phenothiazine derivatives were obtained from

Sigma (St. Louis, MO, USA). HPLC grade methanol and triethylamine were purchased from Fisher (Fair Lawn, NJ, USA). Glacial acetic acid was obtained from Allied Chemical (Pointe Claire, Quebec, Canada). Water was deionized by passing distilled water through a Barnstead water purification system.

3.2.3. Procedures

Mobile phase was prepared by mixing methanol with TEAA buffer. The mobile phase was degassed by bubbling helium through it for about 10 minutes before use. Sample solutions were prepared by dissolving an amount of each compound in methanol to give a concentration of about 1mg/mL. Typically, 2 µl of sample solution was injected. The chromatography was performed at a flow rate of 1.0 ml/min, and the column pressure at this flow rate ranged from 1500 to 2500 psi. Absorbance of the column effluent was monitored at a wavelength of 254 nm.

Almost all of the conventional void volume markers, such as Cl^-, Br^-, I^-, SCN^-, NO_3^-, and MeOH, partially bind to the ß-cyclodextrin-bonded phase column. Consequently, their retention volume cannot be taken to be the column void volume. In this work, the void volume was determined using the following procedure [21]: the retention times (or volumes) of several lower molecular weight analogues of aliphatic n-alcohols were measured on the ß-cyclodextrin column; then, the measured retention data were plotted as solute retention volume vs. the corresponding van der Waals volume, and a linear plot is obtained; the intercept value on the retention volume axis, which corresponds to zero van der Waals volume of the solute, is taken as the column void volume. For the Cyclobond I column (250 x 4.6 mm), the void volume was determined to be 3.05 mL at a flow rate of 1.0 mL/min.

All data points on the graphs were obtained by averaging at least three separate determinations. A reproducibility study involving five injections determined the relative standard deviation of the capacity factor to be < 2%.

3.3. RESULTS AND DISCUSSION

3.3.1. Effect of mobile phase composition.

As stated previously, cyclodextrins form inclusion complexes with various compounds. Although inclusion complex formation can take place in certain organic solvents, such as acetonitrile, methanol, ethanol, dimethylformamide, dimethylsulphoxide, and other dipolar solvents [22], and even in the solid state [23], inclusion selectivity is usually found only in the presence of water. Therefore, an aqueous mobile phase is usually employed in liquid chromatographic separations on cyclodextrin-bonded phase columns.

Preliminary studies of separation conditions showed that most of the compounds of interest cannot be eluted within a reasonable time from the ß-cyclodextrin-bonded phase column by water alone. This indicates that an aqueous-organic eluent might be more suitable. However, when mobile phases consisting of acetonitrile-water or tetrahydrofuran-water were used, poor selectivity was observed, as the capacity factors of the phenothiazine derivatives on the ß-cyclodextrin column were nearly the same. The methanol-water mobile phase was found to provide much better selectivity than acetonitrile-water and tetrahydrofuran-water systems, and was thus chosen as the mobile phase in all subsequent experiments.

The methanol concentration in the mobile phase also affects the values

of the capacity factors on the ß-cyclodextrin stationary phase. The effect of the methanol concentration on the retention was investigated by changing the methanol-water ratio in the mobile phase stepwise from 30:70 to 80:20 (v/v). In this set of experiments, the column temperature was controlled at 20°C, and no TEAA buffer was present in the mobile phase.

Figure 3.1 shows some typical plots of capacity factors versus methanol concentration in the mobile phase. As can be seen from Figure 3.1, both

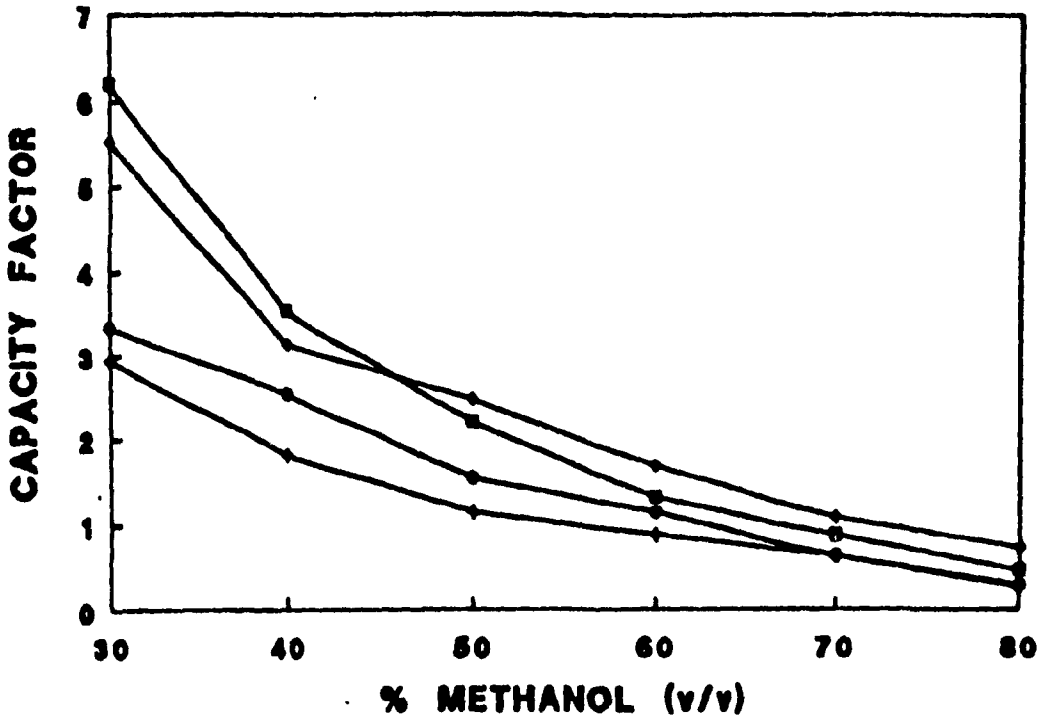


Figure 3.1. Effect of the methanol concentration in the mobile phase on retention. (♦) chloropromazine; (■) acetopromazine; (•) promethazine; (+) propiomazine. Column, Cyclobond I (250 x 4.6 mm); mobile phase, methanol/water (v/v); column temperature, 20°C; flow rate, 1 mL/min.

retention time and selectivity decrease with an increase in the methanol concentration. The effect of changing methanol concentration on the retention time is not linear. When the methanol concentration reaches 80%, there is almost no retention on the ß-cyclodextrin column for most of these compounds. If the logarithm of capacity factors is plotted against the methanol concentration, linear plots, as shown in Figure 3.2, are obtained for most of these compounds, as is the case with most reversed-phase columns [24]. This fact suggests that a reversed-phase mechanism dictates the interaction between the solutes and ß-cyclodextrin stationary phase.

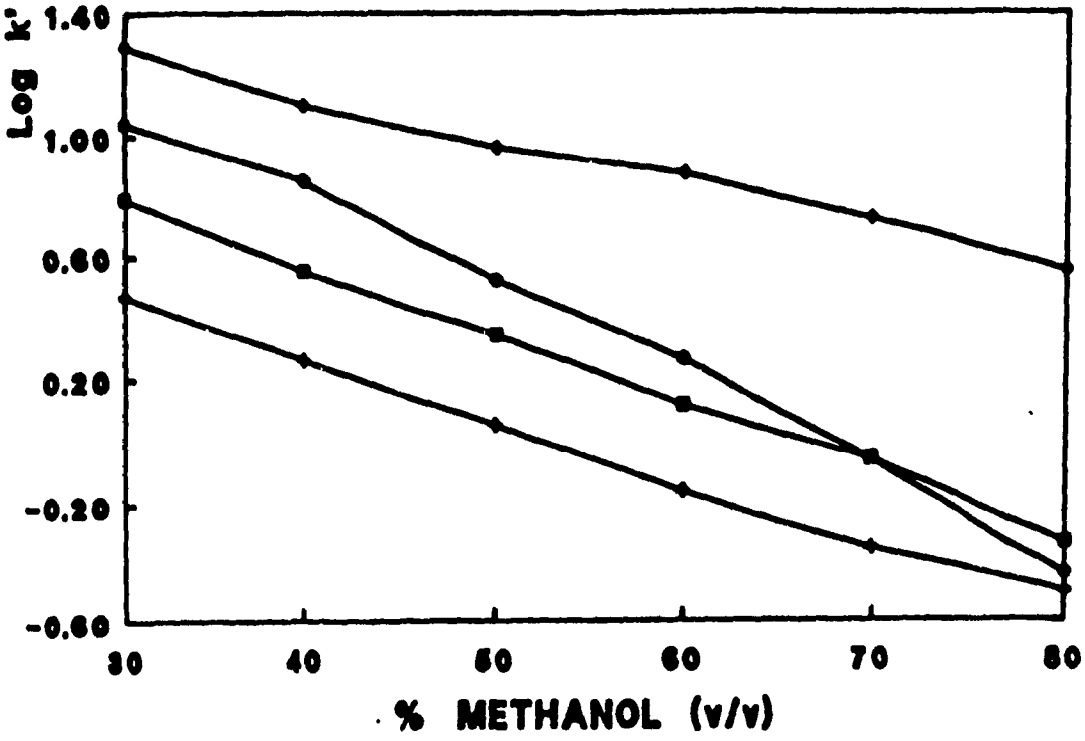


Figure 3.2. Plots of the logarithm of capacity factors versus methanol content. (■) acetopromazine; (♦) prochlorperazine; (•) trimeprazine; (+) 2-(trifluoromethyl)-phenothiazine.

3.3.2. Effect of pH on the retention.

The ß-cyclodextrin bonded phase column is stable over the pH range of 3.5 to 7.5. Therefore, the effect of pH on the retention time of these compounds was investigated by changing the pH of the mobile phase from 3.5 to 7.2. The pH values were obtained by using TEAA buffer (0.02 M), and the temperature was held at 22°C. In this set of experiments, the mobile phase contains 40% methanol.

Table 3.2 lists the capacity factors of these compounds at different pH values as well as those pK_a values that can be found in the literature [2]. The capacity factors for almost all of these compounds are nearly constant within the range of pH investigated. This means that there is no pH effect on retention time over this range. This fact may be rationalized in terms of the pK_a values of these solutes and of ß-cyclodextrin. Although phenothiazine itself has a pK_a value of 2.52, most of the phenothiazine derivatives substituted in the 10- and/or 2-position have pK_a values of 8.10-9.58, and ß-cyclodextrin has a pK_a values of about 12.0 [25]. In the pH range examined, the chemical form of phenothiazine, its derivatives and the ß-cyclodextrin stationary phase cannot be changed. Therefore, the retention time of all of these compounds is unaffected by pH changes.

Table 3.2.

Effect of pH on the retention of phenothiazine and structurally related compounds.

Compounds	Capacity Factors at Different pH values[a]					pK_a[b]
	3.50	4.50	5.50	6.50	7.20	
Acetopromazine	2.20	2.22	2.23	2.27	2.28	--
Chlorpromazine	3.83	3.83	3.86	3.86	3.90	9.30
Promazine	2.83	2.86	2.86	2.86	2.89	9.40
Ethopropazine	1.67	1.70	1.70	1.73	1.73	9.58
Thioridazine	7.43	7.43	7.46	7.46	7.47	9.50
Phenothiazine	2.57	2.57	2.60	2.63	2.63	2.52
Prochlorperazine	10.54	10.57	10.60	10.63	10.69	8.10
Promethazine	1.87	1.89	1.93	1.96	1.96	9.10
Propiomazine	1.67	1.71	1.74	1.74	1.77	--
Perphenazine	6.60	6.63	6.66	6.70	6.73	--
Trifluoperazine	6.80	6.83	6.83	6.86	6.89	8.10
Trimeprazine	2.70	2.70	2.73	2.76	2.76	9.00
2-methoxyphenothiazine	2.93	2.96	2.99	3.02	3.02	--
2-acetylphenothiazine	2.17	2.20	2.20	2.23	2.23	--
Triflupromazine	2.37	2.40	2.40	2.42	2.43	9.41
2-(Trifluoromethyl)-phenothiazine	2.70	2.73	2.76	2.76	2.81	--

[a]Conditions: column, Cyclobond I; mobile phase, 40 : 60 methanol/ 0.02 M TEAA buffer; T = 22°C; flow rate, 1 ml/min. [b]Data from reference [2].

3.3.3 Effect of TEAA buffer concentration.

The effect of TEAA concentration on the retention is studied by varying TEAA concentration from 0.0 to 0.10 M. In this set of experiments, the mobile phase contained 40% methanol, the pH was maintained at 5.0, and the column temperature was set at 22°C. The capacity factors of these compounds at different TEAA concentrations are listed in Table 3.3. The retention times of all the compounds decreased with the addition of TEAA buffer and more or less stabilized at TEAA > 0.01 M. This type of retention behaviour was previously encountered with the retention behaviour of chlorophenols and chlorobiphenols as well [19,20].

It was also observed that the column efficiency was substantially increased with the addition of TEAA buffer to the mobile phase. For these compounds, a TEAA solution (0.02 M, pH 5.0) substituted for water in a methanol/water (40:60) system produces a three- to four-fold increase in the column efficiency. The selectivity of the separation was not affected in this case, as no changes were observed in the elution order of the compounds with increasing TEAA concentration. Since the presence of TEAA in the mobile phase does not decrease the selectivity, the ß-cyclodextrin bonded phase column can be coupled with electrochemical detectors. As an electrolyte, TEAA increases the conductivity and minimizes the iR drop between the reference and working electrodes, thus facilitating the efficient operation of an electrochemical detector.

Table 3.3.

Effect of TEAA buffer concentration on the retention of phenothiazine and structurally related compounds

Compounds	Capacity Factors at Different TEAA Concentration[a]				
	0.00	0.01	0.02	0.05	0.10
Acetopromazine	3.53	2.26	2.23	2.22	2.18
Chlorpromazine	4.60	3.93	3.86	3.86	3.83
Promazine	3.13	2.86	2.86	2.86	2.83
Ethopropazine	1.93	1.75	1.74	1.72	1.72
Thioridazine	8.33	7.49	7.46	7.46	7.45
Phenothiazine	3.00	2.63	2.60	2.57	2.54
Prochlorperazine	12.67	10.72	10.60	9.90	9.55
Promethazine	2.53	2.00	1.73	1.67	1.58
Propiomazine	1.80	1.77	1.74	1.68	1.59
Perphenazine	6.80	6.75	6.66	6.33	6.03
Trifluoperazine	7.06	5.56	5.33	5.27	5.17
Trimeprazine	2.79	2.76	2.73	2.63	2.51
2-methoxyphenothiazine	3.27	3.05	2.99	2.80	2.56
2-acetylphenothiazine	2.27	2.26	2.20	1.93	1.73
Triflupromazine	2.43	2.33	2.33	2.26	2.22
2-Trifluoromethyl-phenothiazine	2.80	2.76	2.47	2.35	2.28

[a]Conditions: column, Cyclobond I; mobile phase, 40% methanol; pH = 5.0; T = 22°C.

3.3.4. Effect of temperature

The effect of column temperature on the retention was examined by changing the temperature from 20 to 60°C with a mobile phase of methanol-water (50:50, v/v). Changes in temperature were seen to have a substantial effect on the retention of the solute on the ß-cyclodextrin column. The retention times of these compounds all decrease with increasing temperature. When the column temperature was increased to 60°C, there was almost no retention at all for most of these compounds. These results indicate that the binding constant of the solute to ß-cyclodextrin decreases with increasing temperature and that inclusion formation is effectively prevented for most solutes at a temperature higher than 60°C.

van't Hoff plots (Figure 3.3), the plots of the logarithm of capacity factors versus the reciprocal of the absolute temperature, are nonlinear for most of these compounds. According to Horvath [26], nonlinear van't Hoff plots can be expected whenever one of the following three conditions holds: (i) the eluate exists in two or more forms having different retention; (ii) there exist two or more retention mechanisms due to the heterogeneity of the stationary phase surface containing more than one binding site; or (iii) the eluate exists in more than one form and the surface is heterogeneous. The studies of Otagiri *et al.* [27] suggest that the interaction of ß-cyclodextrin with phenothiazine and its derivatives takes place at different bonding sites; the aromatic portion of phenothiazine drugs is incorporated into the ß-cyclodextrin cavity through hydrophobic interactions while the N-substituents of the drug interact with the outside surface of the cavity by hydrogen bonding. Thus, the nonlinear van't Hoff plots observed in this study are likely due to condition (ii) being in effect.

The selectivity is only slightly affected by changing temperature. In this respect, the ß-cyclodextrin column appears to behave like other reversed-phase columns [28].

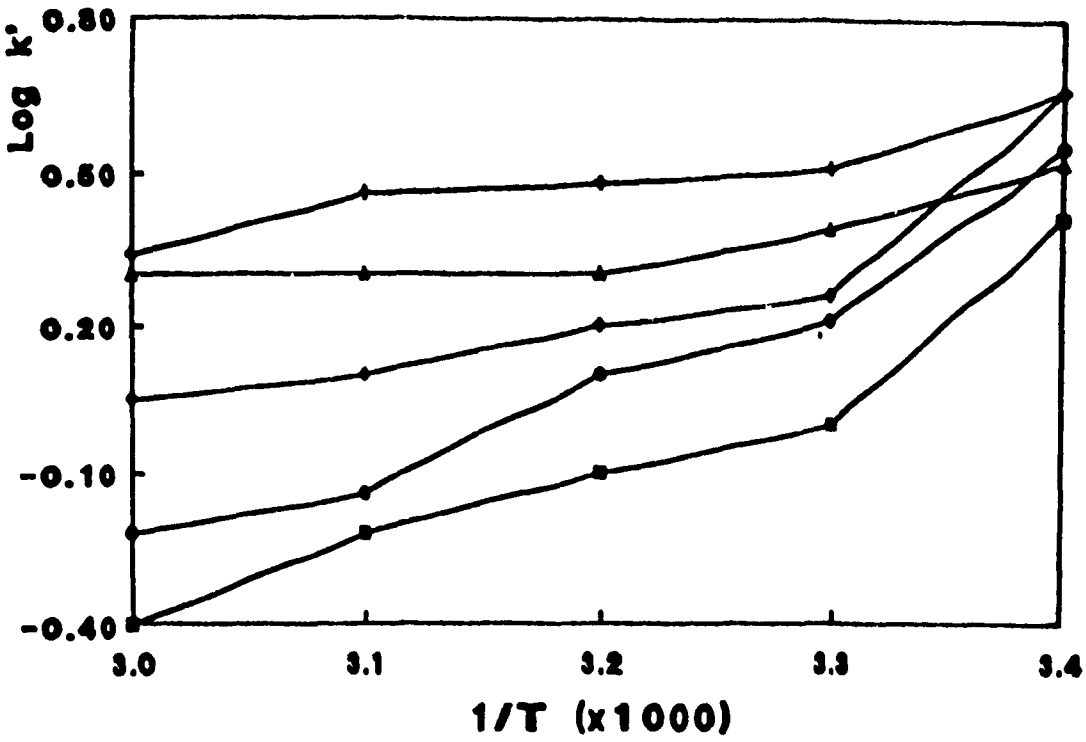


Figure 3.3. van't Hoff plots of some studied conpounds. (■) 2-acetylphenothiazine; (♦) chlorpromazine; (•) promazine; (▲) trifluoperazine; (+) perphenazine; column, Cyclobond I; mobile phase, 50:50 methanol/water; column temperature, 22°C; flow rate, 1 mL/min.

3.3.4. Separations

After studying the liquid chromatographic retention behaviour of these compounds on the ß-cyclodextrin-bonded phase column with respect to mobile phase composition, pH, TEAA buffer concentration and column temperature, the following isocratic conditions are chosen for the separation of these compounds: methanol-water (35:65, v/v); 0.05 M TEAA; pH 4.5; and T = 20°C. Under these conditions, all 16 compounds eluted within a reasonable time (43 min.). Figure 3.4 shows the chromatogram obtained under these isocratic

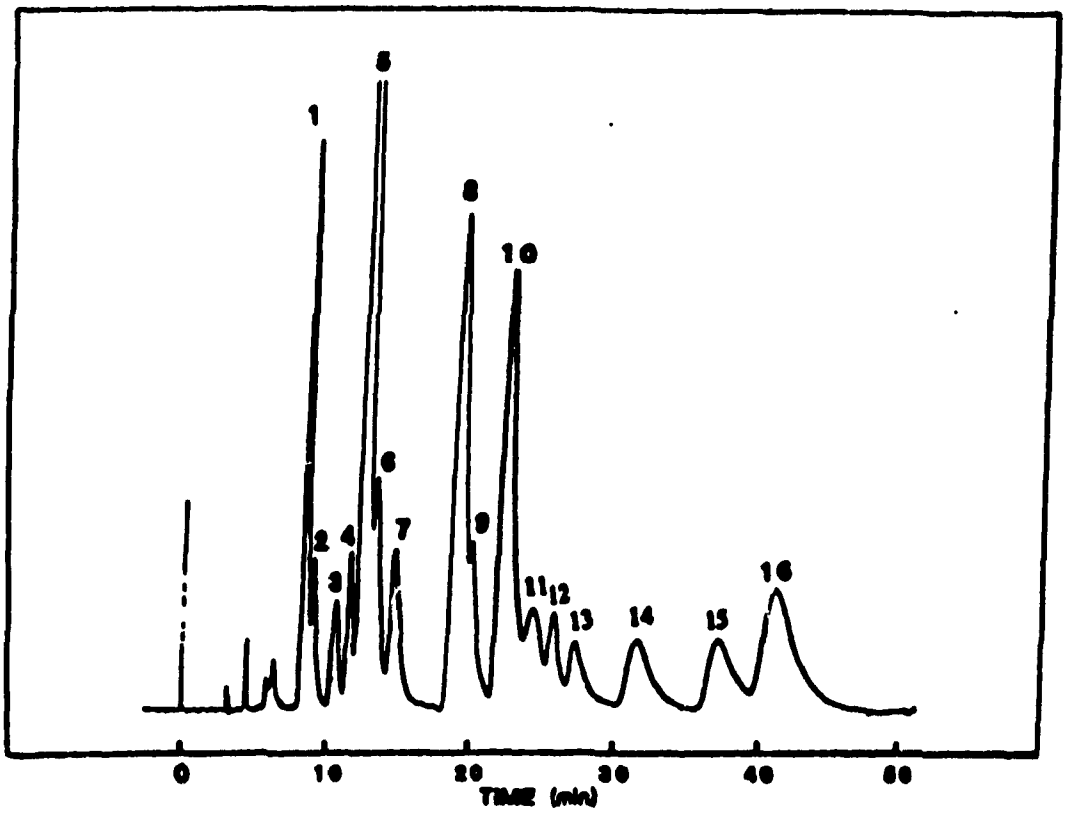


Figure 3.4. Isocratic separation of phenothiazines and structurally related drugs. Peaks identification: (1) propiomazine; (2) ethopropazine; (3) promethazine; (4) 2-acetylphenothiazine; (5) acetopromazine; (6) triflupromazine; (7) 2-(trifluoromethyl)-phenothiazine; (8) promazine; (9) phenothiazine; (10) trimeprazine; (11) 2-methoxylphenothiazine; (12) Chlorpromazine; (13) trifluoperazine; (14) perphenazine; (15) thioridazine; (16) prochlorperazine. Conditions: column, Cyclobond I (250 x 4.6 mm); mobile phase, 35:65 methanol/TEAA buffer (0,05 M TEAA, pH = 4.5); column temperature, 20°C; flow rate, 1 mL/min.

conditions. As can be seen from the chromatogram, most of the 16 compounds can be separated with reasonable resolution. However, because a certain degree of overlap existed for these compounds, the complete separation using an isocratic elution technique is impossible.

In order to achieve the complete separation of all of the compounds, a gradient elution technique is used. Figure 3.5 shows the chromatogram achieved with a linear mobile phase gradient. The separation is carried out at an initial condition of 30:70 of solvent A (methanol) to solvent B (0.05 M TEAA buffer solution, pH 4.1) with a linear increase of 0.5% solvent A per minute. As can be seen from Figure 3.5, the resolution is much improved using this gradient elution system.

Previous experience with gradient elution on a ß-cyclodextrin-bonded phase column has shown that the steepness of the gradient must be less than what is conventionally used in reversed-phase chromatography with C_{18} columns. In this gradient elution separation, if the steepness is greater than a 1% min^{-1} increase in methanol, no separation can be observed. This result indicates that the separation is governed by equilibria involving the free and bonded forms of the solute as well as solvent molecules going from or to the cyclodextrin cavity; the slow kinetics of these equilibria dictates a limit to the steepness of the gradient.

In conclusion, this investigation has demonstrated that the ß-cyclodextrin-bonded phase column is very selective toward phenothiazine and its structurally related derivatives. Although the separation of these compounds can be achieved by an isocratic elution mode, a better resolution is achieved with the use of a gradient method.

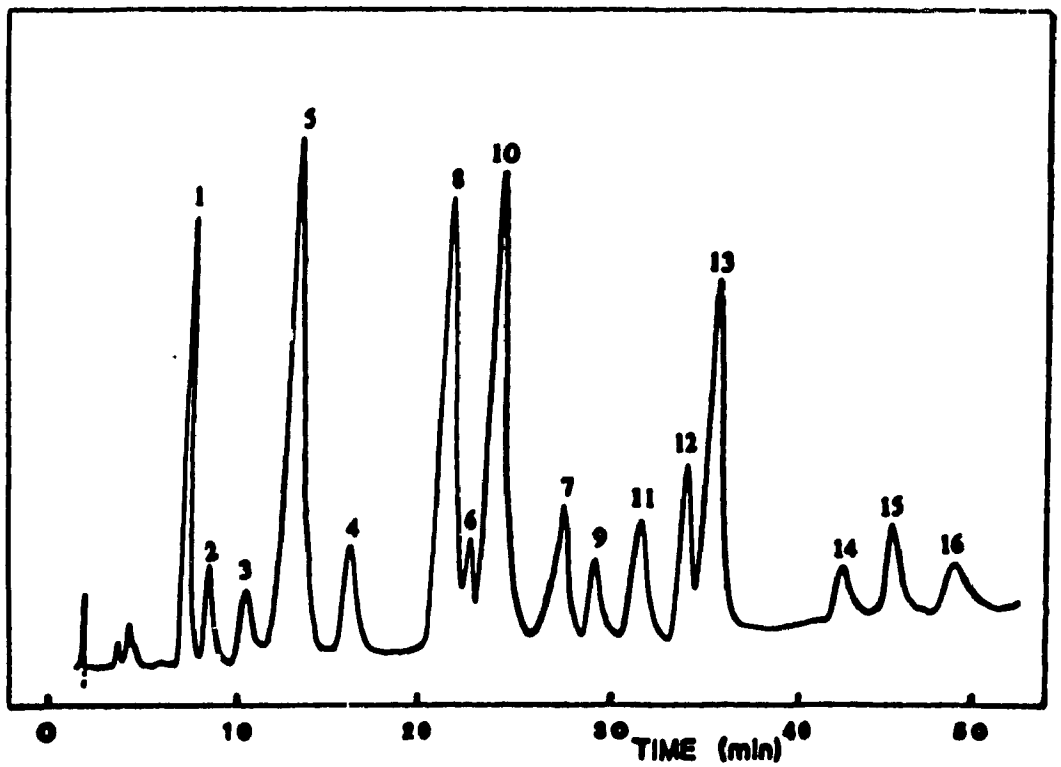


Figure 3.5. Gradient elution separation of phenothiazines and structurally related drugs. Peaks identification: (1) propiomazine; (2) ethopropazine; (3) promethazine; (4) 2-acetylphenothiazine; (5) acetopromazine; (6) trifluprομazine; (7) 2-(trifluoromethyl)-phenothiazine; (8) promazine; (9) phenothiazine; (10) trimeprazine; (11) 2-methoxylphenothiazine; (12) Chlorpromazine; (13) trifluoperazine; (14) perphenazine; (15) thioridazine; (16) prochlorperazine. Conditions: column, Cyclobond I (250 x 4.6 mm); mobile phase gradient starts at 30% of solvent A (methanol) and 70% solvent B (0.05 M TEAA buffer solution, pH = 4.1) with linear gradient of 0.5%/min increase in solvent A; column temperature, 20°C; the total flow rate, 1 mL/min; detector UV at 254 nm.

3.4. REFERENCES

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Chapter 4

LIQUID CROMATOGRAPHIC SEPARATION OF THE ENANTIOMERS OF AMINO ACIDS AS THEIR DINITROPHENYL DERIVATIVES ON A ß-CYCLODEXTRIN-BONDED PHASE COLUMN

4.1. INTRODUCTION

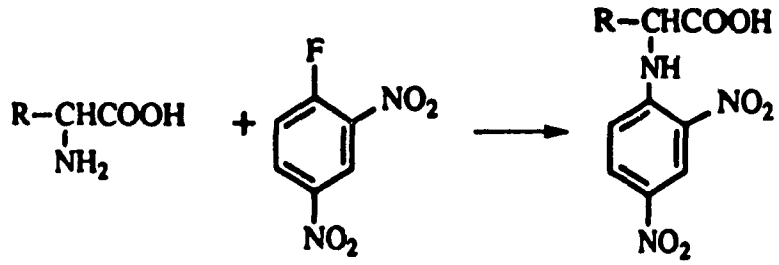
Enantiomeric separation of amino acids is of importance in many fields, such as peptide synthesis, asymmetric syntheses in organic chemistry, amino acid biochemistry, the studies of food processing and protein degradation processes in humans [1-3], and dating of archaeological materials [4]. For the last several years, there existed two general approaches to the high-performance liquid chromatographic separations of the enantiomers of amino acids. One is ligand-exchange chromatography using a chiral ligand immobilized on a solid support as the stationary phase. The other technique employs active chelates in the mobile phase. Recently, cyclodextrin-bonded stationary phases developed by Armstrong and coworkers [5, 6] were also successfully used for the enantiomeric resolution of amino acids.

As mentioned in Chapter 1, cyclodextrins are oligosaccharides in which glucose units are joined together to form a toroidal structure with a hydrophobic cavity and hydrophillic exterior faces. The main property of cyclodextrins which allows them to affect chiral separation is their ability to

form enantioselective complexes with chiral guest molecules. Although little experimental effort has been focused on a detailed explanation of the nature of chiral discrimination interactions and chirality forces responsible for the different retentions of the enantiomers within the stationary phase, a number of suggestions of chiral recognition mechanism have been made. It is believed that chiral recognition is caused by inclusion complex formation between the cyclodextrin cavity and the hydrophobic moiety of the solute, and by hydrogen bonding between the polar functional groups of the solute in the vicinity of its chiral center and the hydroxyl groups of the cyclodextrin [5].

The enantiomers of tryptophan, phenylalanine, tyrosine and some other aromatic amino acids have been separated on an α-cyclodextrin-bonded phase column [6]. To date, however, there has been no report on the enantiomeric separation of non-aromatic amino acids, although the separations of some racemic amino acids as 5-dimethylamino-1-naphthalenesulphonyl (dansyl) derivatives using a ß-CD bonded phase column have also been reported [5].

In this chapter, a new approach for the enantiomeric separations of racemic amino acids is described. In this approach, the racemic non-aromatic amino acids are first derivatized to dinitrophenyl (DNP) amino acids by reacting them with fluoro-2,4-dinitrobenzene (FDNB):



The racemic DNP-amino acids are then separated using a ß-cyclodextrin-

bonded stationary phase column with traditional aqueous-organic mobile phases. The effects of pH, the mobile phase composition, buffer concentration and the structural features of the solutes on the retention time and enantiomeric resolution are discussed in terms of the retention mechanism.

4.2 EXPERIMENTAL

4.2.1 Apparatus

The liquid chromatographic system was described in section 3.2.1.

4.2.2 Chemicals

DNP-D-valine, DNP-D-leucine and DNP-DL-methionine were synthesed by modifying the preparation procedure described by Schroeder and Legette [7] (see section 4.2.3). All other D- and L-DNP-amino acids and fluoro-2,4-dinitrobenzene (FDNB) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HPLC-grade methanol and triethylamine were purchased from Fisher (Fair Lawn, NJ, U.S.A.). Glacial acetic acid was obtained from Allied Chemical (Pointe Claire, Canada). Water was deionized by passing distilled water through a Barnstead water-purification system.

4.2.3. Preparation of DNP-amino acids

Twenty mL of 1.0 M $NaHCO_3$ solution was transferred to a 150-ml glass-stoppered flask followed by additions of a magnetic stirring bar, 0.01 mole of amino acid (solid), and 20 ml of FDNB-ethanol solution (containing 2.0 g of FDNB). The mixture was then stirred at room temperature for 3 hours. After

evaporation of the ethanol, the residue was neutralized to pH 9.0 with 6 N HCl. This solution was then transferred to a 200 ml separatory funnel with 20 ml of water. The solution was extracted six times with 30 ml of ether to remove the excess FDNB. After removal of the excess FDNB, the aqueous phase was adjusted to pH 1.5. DNP-amino acids were extracted with ether and dried for identification and application.

For DNP-D-valine, the product weight was 1.5 g (yield 53%). ^{1}H NMR (in D_2O, pH 11.0, 0.05 M phosphorate buffer) gave: 8.96 (3H, aromatic), 8.10 (5H, aromatic), 6.78 (6H, aromatic), 3.90 (α-CH-), 2.15 (ß-CH-), and 0.90 (-CH_3); UV maxima (in 0.01 M phosphorate buffer, pH = 6.0): 264.4 nm and 361.8 nm.

For DNP-D-leucine, the product weight was 1.3 g (yield 44%), ^{1}H NMR (in D_2O, pH 11.0, 0.05 M phosphorate buffer) gave: 8.97 (3H, aromatic), 8.11 (5H, aromatic), 6.75 (6H, aromatic), 4.10 (α-CH-), 3.18 (ß-CH_2-), 1.70 (γ-CH-), and 0.81 (-CH_3); UV maxima: 263.1 nm and 362.8 nm.

For DNP-D-methionine and DNP-L-methionine, the product weights were 1.5 g (yield 47%) and 1.4 g (yield 44%), respectively. Both enantiomers had the same NMR, UV and IR spectra at the measuring conditions. ^{1}H NMR (in D_2O, pH 11.0, 0.05 M phosphorate buffer) gave: 8.89 (3H, aromatic), 8.10 (5H, aromatic), 6.76 (6H, aromatic), 4.18 (α-CH-), 2.08 (ß-CH_2-), 2.46 (SCH_2-), and 1.92 (-CH_3). UV maxima: 264.1 nm and 361.5 nm.

4.2.4 Procedures

The mobile phase was prepared by mixing methanol with triethylammonium acetate (TEAA) buffer. The mobile phase was degassed by bubbling helium into it for about 10 min before use. Sample solutions were

prepared by dissolving each raceme in methanol to give a concentration of about 1 mg/ml. Typically, 2 µl of sample solution were injected. The chromatography was performed at a flow-rate of 1.0 ml/min. Absorbance of the column effluent was monitored at a wavelength of 254 nm.

All the data points on graphs were obtained by averaging at least three separate determinations. A reproducibility study involving five injections revealed a relative standard deviation of less than 2% in capacity factors, and of less than 6% in resolution factors.

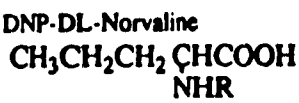
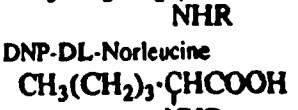
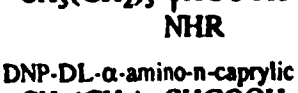
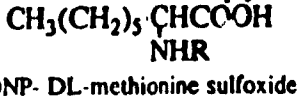
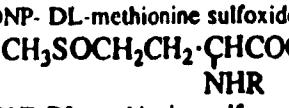
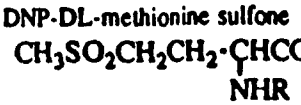
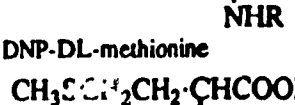
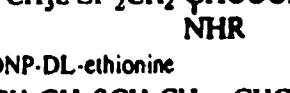
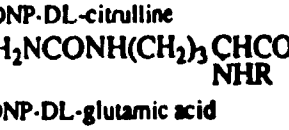
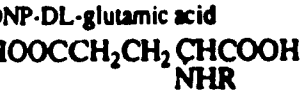
4.3 RESULTS AND DISCUSSION

In this investigation, the optical resolutions of 12 pairs of DNP-amino acid enantiomers are examined using a ß-cyclodextrin-bonded phase column. Table 4.1 lists the optical separation data obtained, together with the structures of these amino acid derivatives. The D-enantiomers eluted first for all the DNP-derivatized amino acids. Some typical chromatograms are shown in Fig. 4.1. As can be seen, the ß-cyclodextrin column exhibits high enantioselectivity toward the DNP-amino acids. The enantiomers of these DNP-amino acids could be separated on a 250 x 4.6 mm I.D. ß-cyclodextrin-bonded phase column with resolution factors from 0.60 up to 3.40.

4.3.1 Effect of structural features on enantioselectivity

From our experience of working with a ß-cyclodextrin-bonded phase column and to the best of our knowledge, enantiomers of underivatized amino acids cannot be separated with the ß-cyclodextrin column. It is believed that the size of the unsubstituted amino acids is too small to bind tightly with the cyclodextrin cavity to form a strong inclusion complex [8, 9], a prerequisite for chiral recognition. The results of this study show that the DNP substituent of

Table 4.1. Optical resolution of the enantiomers of DNP amino acids[a]

No.	solutes[b]	k'[c]	α	R_s	mobile phase[d]
1.	DNP-DL-α-amino-n-butyrc acid 	3.00	1.04	0.60	20:80
2.	DNP-DL-Norvaline 	4.0	1.06	0.80	10:90
3.	DNP-DL-Norleucine 	2.67	1.28	2.45	25:75
4.	DNP-DL-α-amino-n-caprylic acid 	11.3	1.30	3.40	25:75
5.	DNP- DL-methionine sulfoxide 	6.67	1.05	0.8	5:95
6.	DNP-DL-methionine sulfone 	1.32	1.05	0.9	5:95
7.	DNP-DL-methionine 	1.88	1.14	1.50	25:75
8.	DNP-DL-ethionine 	5.00	1.18	2.50	25:75
9.	DNP-DL-citrulline 	1.90	1.05	0.80	5:95
10.	DNP-DL-glutamic acid 	3.60	1.06	0.90	10:90
11.	DNP-DL-valine 	6.81	1.18	2.45	30:70
12.	DNP-DL-leucine 	11.38	1.53	2.75	30:70

a. The separations were done on a 250 x 4.6 mm β-cyclodextrin bonded phase column

b. R in the structures represent the 2,4-dinitrophenyl group.

c. The capacity factor of the first eluted enantiomer.

d. The numbers represent the volume ratio of methanol to TEAA buffer (0.5%, pH 6.20)

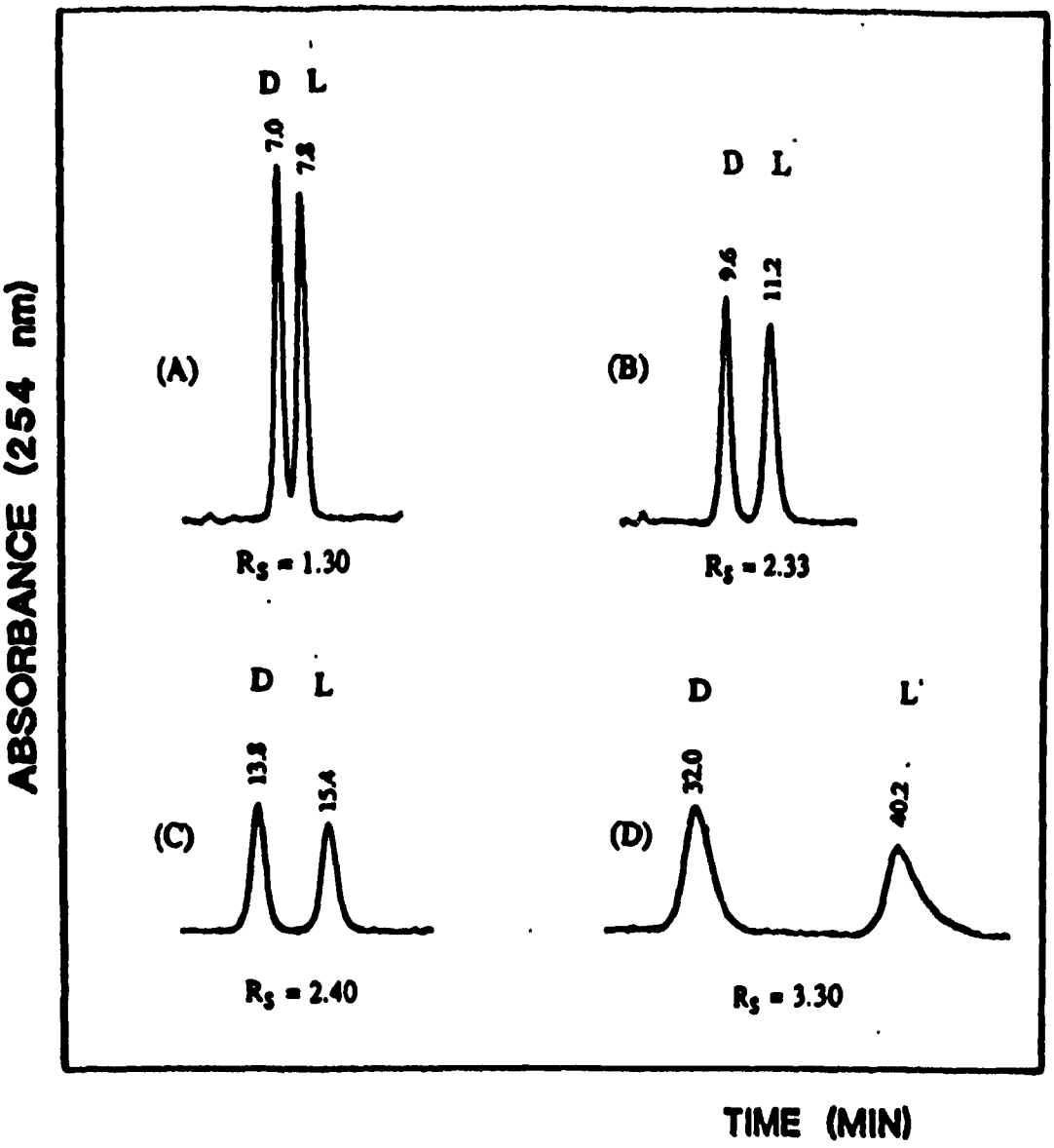


Figure 4.1. Chromatograms showing the resolutions of DNP-amino acid enantiomers: (A) DNP-DL-methionine; (B) DNP-DL-norleucine; (C) DNP-DL-ethionine; and (D) DNP-DL-α-amino-n-caprylic acid. Column, Cyclobond I (250 x 4.6 mm); mobile phase, 30:70 methanol/TEAA buffer (0.5%, pH 6.2); temperature, 20°C; flow rate, 1 mL/min.

the derivatized amino acids plays an important role in chiral recognition. This is not surprising since it had been previously reported that the nitrophenyl groups could tightly bind to the ß-cyclodextrin cavity to form a strong inclusion complex [10, 11]. The introduction of a DNP substituent into the amino acid molecules provides the strong binding site required for the chiral recognition.

A comparison of the enantioselectivity (α) values obtained for the DNP amino acids indicates that the enantioselectivity is also affected by the size of the alkyl substituents around the chiral center. As can be seen from Table 4.1, the enantioselectivities (α) for α-amino-n-butyric acid, norvaline, norleucine and α-amino-n-caprylic acid are 1.04, 1.06, 1.28 and 1.30, respectively. The only difference between the structures of these solutes is the size of the alkyl substituent on the chiral center. These results indicate that the enantioselectivity increases with the size of alkyl substitent. This is true for the enantioselectivities of DNP-DL-methionine (α = 1.14) and DNP-DL-ethionine (α = 1.18), and DNP-DL-valine (α = 1.18) and DNP-DL-leucine (α = 1.53) as well. It seems that the alkyl substituent plays an important role of steric hindrance, which weakens the strength of inclusion complexation and/or hydrogen bonding for one of the enantiomers.

4.3.2 Effect of mobile phase composition

As discussed in Chapter 3, with the use of an aqueous methanol solution as mobile phase, the ß-cyclodextrin-bonded phase column provides the best selectivities for the separation of structurally related compounds. This is true for the resolution of racemic DNP-amino acids as well. Preliminary studies of separation conditions show that when acetonitrile/water and tetrahydrofuran/water systems were used as the mobile phase, no resolution can be observed for these racemic DNP-amino acids. Methanol/water is found to provide much better enantioselectivity than acetonitrile/water and tetrahydrofuran/water systems. Therefore, a methanol/water mixture is used as the mobile phase for the separations.

4.3.3 Effect of methanol content

The effects of the methanol content on the retention and resolution were investigated by changing the methanol-water ratio in the mobile phase. Figure 4.2 and Figure 4.3 show the typical plots of capacity factors and resolution factors versus the methanol contents, respectively. The TEAA buffer concentration was 0.5%, and the pH of the mobile phase was 5.0 in this set of experiments.

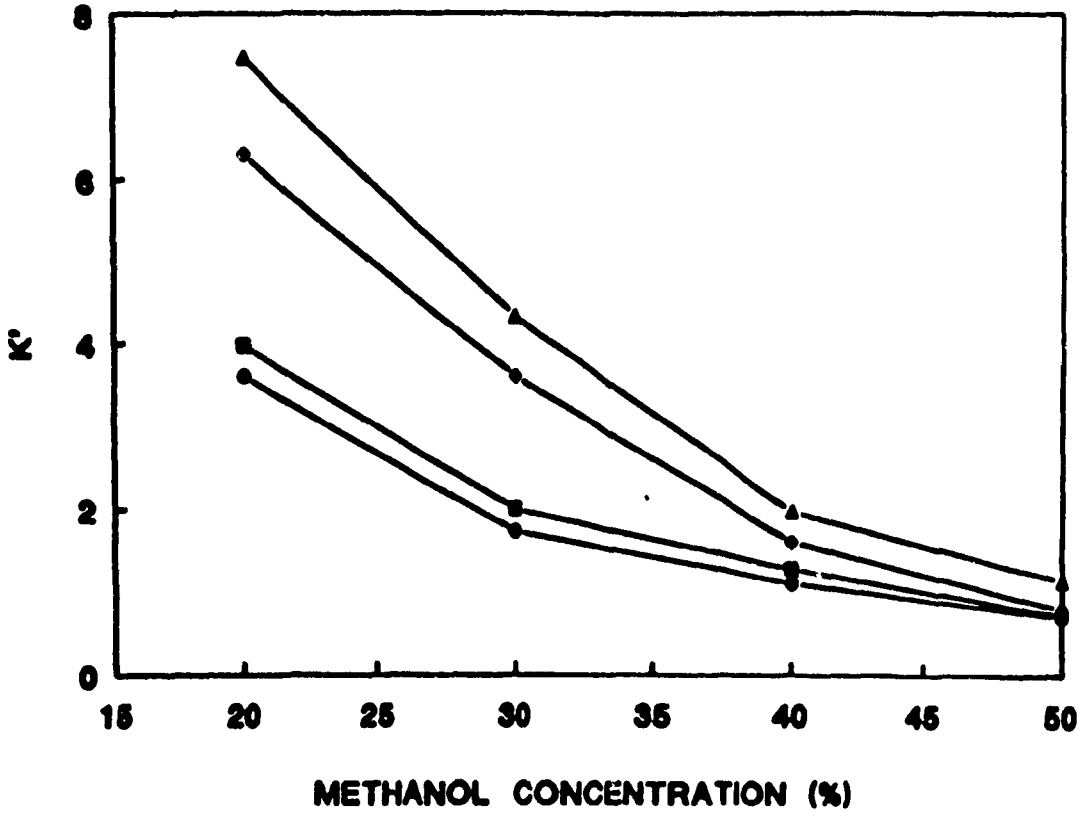


Figure 4.2. Effect of methanol concentration in the mobile phase on the retention of (▲) DNP-DL-ethionine; (●) DNP-DL-α-amino-n-caprylic acid; (■) DNP-DL-methionine; (◆) DNP-DL-norleucine. Column, Cyclobond I (250 x 4.6 mm); mobile phase, methanol-TEAA buffer (0.5% TEAA); pH, 5.0; temperature, 20°C; flow rate, 1 mL/min. k' is the capacity factor of the first eluted enantiomer.

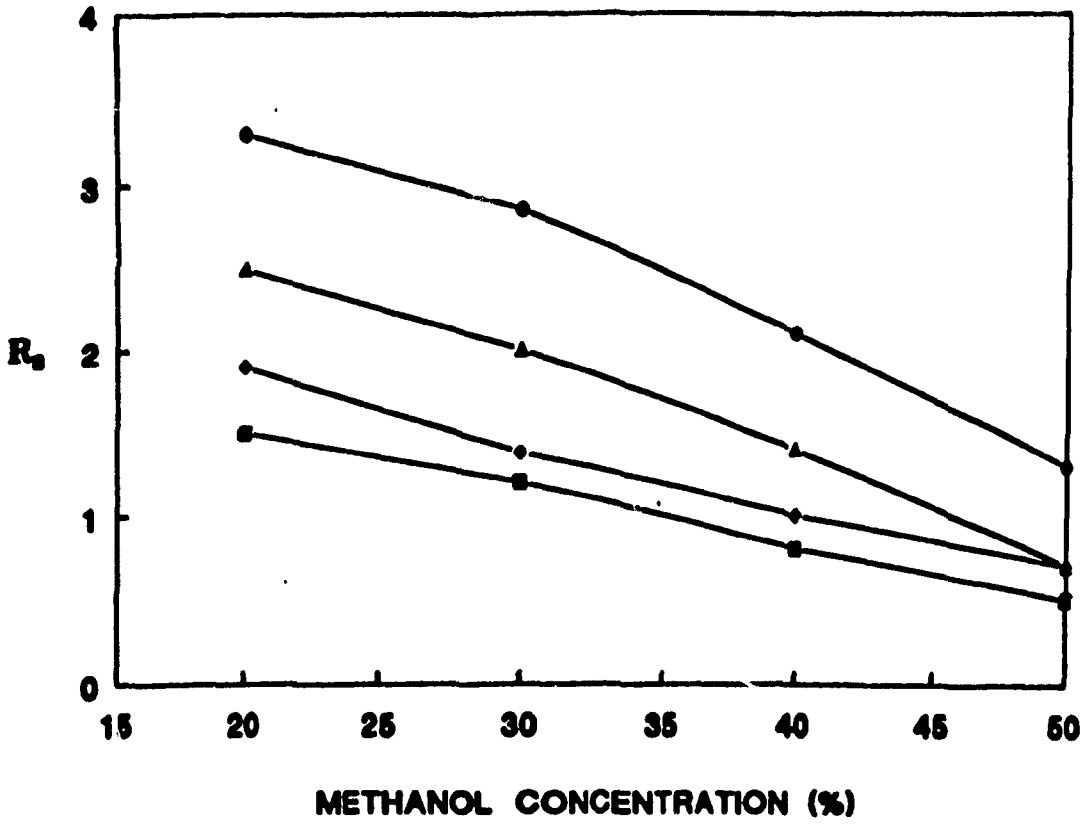


Figure 4.3. Effect of methanol concentration in the mobile phase on the resolution of (▲) DNP-DL-ethionine; (●) DNP-DL-α-amino-n-caprylic acid; (■) DNP-DL-methionine; (◆) DNP-DL-norleucine. Column, Cyclobond I (250 x 4.6 mm); mobile phase, methanol-TEAA buffer (0.3% TEAA); pH, 5.0; temperature, 20°C; flow rate, 1 mL/min.

It was found that the effect of methanol concentration on the retention and enantiomeric resolution of DNP-amino acids gave almost the same tendencies as those observed with dansyl-amino acids [5], that is, an increase in the methanol content resulted in both a decreased retention and a decreased enantioelectivity and resolution factor. For some DNP-amino acids, such as DNP-DL-methionine sulfoxide and DNP-DL-citrulline, the enantiomeric resolution could be achieved only at very low methanol concentration (<10%).

This is not surprising since it is known from the cyclodextrin-binding studies that methanol and other alcohols, such as ethanol, propyl alcohol, and n-butyl alcohol can form inclusion complexes with ß-cyclodextrin [12]. The formation constant of the 1:1 ß-cyclodextrin-methanol complex has been measured by spectrophotometric examination of the effect of methanol on the association of ß-cyclodextrin with sodium 4-[(4-hydroxy-1-naphthyl)azo]-1-naphthalene-sulfonate and phenolphthalein [13, 14], respectively. Assocation constants of 0.32 and 0.40 M^{-1} were reported. Assuming that the same considerations apply to our system, the following simple complexation pattern can be used to explain the results obtained:

$$G + CD = G\text{-}CD \qquad (1)$$

$$K = [G\text{-}CD]/[G]\{CD] \qquad (2)$$

$$Me + CD = Me\text{-}CD \qquad (3)$$

$$K_{MeCD} = [Me\text{-}CD]/[Me]\{CD] \qquad (4)$$

where CD, G, and Me denote ß-CD, DNP-amino acids, and methanol, respectively. G-CD and Me-CD are the inclusion complexes of ß-cyclodextrin-DNP-amino acid and cyclodextrin-methanol, respectively. K and K_{MeCD} are the corresponding complex formation constants. Therefore, the true formation constant (K^*) for the inclusion complex of cyclodextrin with DNP-amino acid can be expressed as

$$K^* = \frac{K}{1 + K_{MeCD} \cdot [Me]} \qquad (5)$$

If the possible formation of higher stoichiometries between ß-cyclodextrin and methanol is taken into consideration, the following equation (4) can be derived:

$$K' = \frac{K}{1 + K_{MeCD} \cdot [Me] + K_{MeCD} \cdot [Me]^2 + \ldots} \qquad (6)$$

As can be seen from equation (6), at higher methanol concentrations, methanol can become strongly competitive for complexation with ß-cyclodextrin thereby decreasing the degree of complexation between DNP-amino acid and ß-cyclodextrin. In addition, at high methanol concentrations, the properties of the bulk solvent begin to change substantially. Consequently, the presence of the methanol will likely make the solvent more favorable to DNP-amino acids than a simple aqueous solution. The difference in hydrophobicity of the solvent and ß-cyclodextrin cavity will become smaller, making DNP-amino acid complexation with ß-cyclodextrin less favorable. Both of these effects could account for the decreases in retention time and resolution factor when the methanol content in the mixture is increased.

4.3.4 Effect of TEAA buffer concentration

Fig. 4.4 shows the influence of TEAA buffer concentration in the mobile phase on the retention. It is found that an increase in the TEAA concentration in the mobile phase results in a decreased retention of both enantiomers in all instances. However, the effect of TEAA concentration on the enantiomeric resolution factors is somewhat more complex (see Figure 4.5). As the TEAA concentrations in the mobile phase vary from 0.1% to 1.0%, two types of

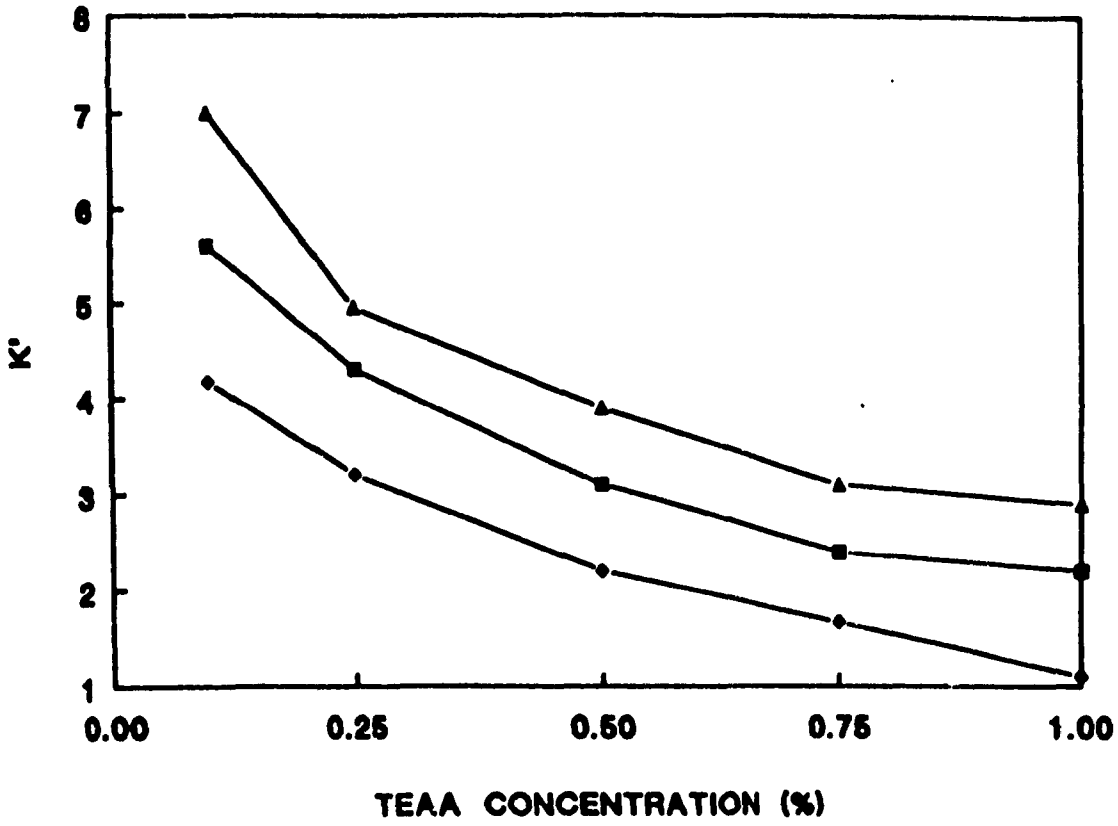


Figure 4.4. Effect of TEAA concentration in the mobile phase on the retention of (▲) DNP-DL-ethionine; (■) DNP-DL-norleucine; (♦) DNP-DL-methionine. Column, Cyclobond I (250 x 4.6 mm); mobile phase, methanol-TEAA buffer (30% methanol); pH, 5.0; temperature, 20°C; flow rate, 1 mL/min. k' is the capacity factor of the first eluted enantiomer.

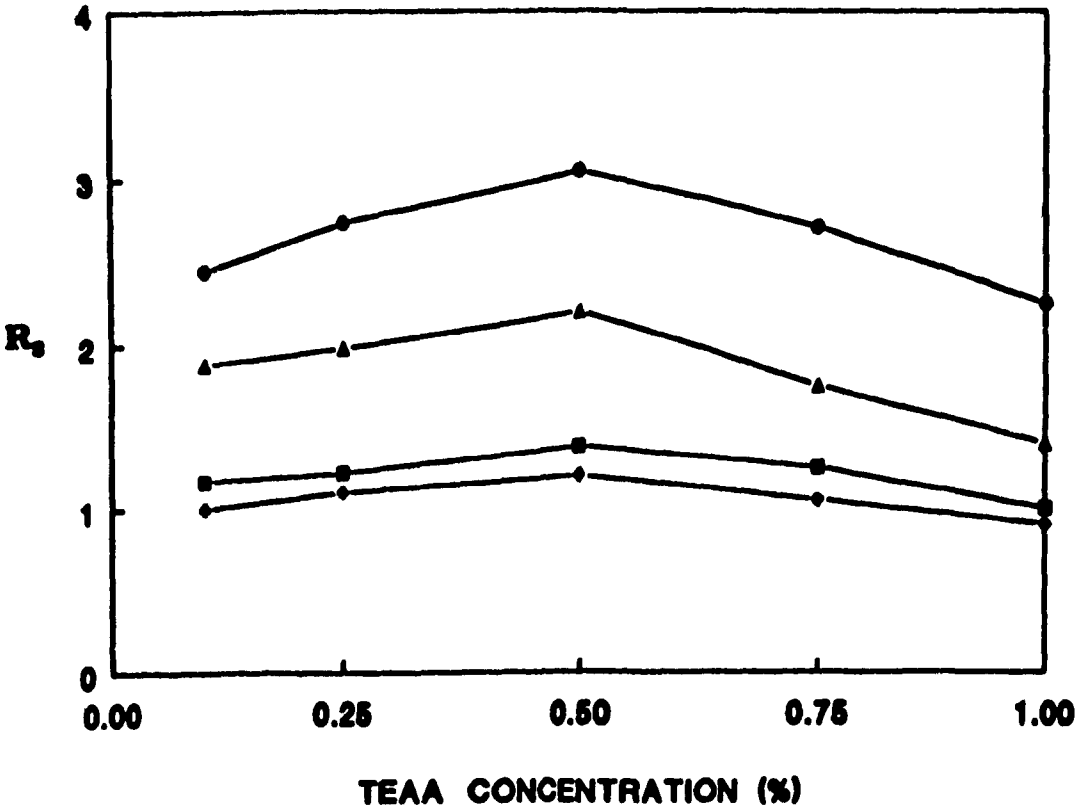


Figure 4.5. Effect of TEAA concentration in the mobile phase on the resolution of (▲) DNP-DL-ethionine; (●) DNP-DL-α-amino-n-caprylic acid; (■) DNP-DL-methionine; (◆) DNP-DL-norleucine. Column, Cyclobond I (250 x 4.6 mm); mobile phase, methanol-TEAA buffer (30% methanol); pH, 5.0; temperature, 20°C; flow rate, 1 mL/min.

behaviour can be observed. The resolution first increases with the increasing TEAA concentration and then decreases when TEAA is greater than 0.5%. Resolution maxima are observed at TEAA concentrations of about 0.5%.

These facts can be explained by considering the effect of TEAA on both the column efficiency (N) and the enantioselectivity (α) of ß-CD bonded phase column, and the relationship of resolution (R_s), N and α:

$$R_s = \frac{1}{4} (\alpha - 1) \sqrt{N} \frac{k'}{(1 + K')} \qquad (7)$$

It has been found that the addition of TEAA buffer in the mobile phase will substantially increase the separation efficiency of a ß-cyclodextrin bonded phase column. As was presented in Chapter 3, a TEAA buffer (0.02 M and pH 5.0) substituted for water in a methanol-water (40:60) system can produce a three- to four-fold increase in the column efficiency for the separation of phenothiazine derivatives [15]. The increase in separation efficiency will result in an increase in the resolution factor.

On the other hand, the TEAA molecule, as an organic modifier, can include in the ß-cyclodextrin cavity and there it competes with solute. The addition of TEAA in the mobile phase will weaken the strength of inclusion complexation between the DNP substituent and the ß-cyclodextrin cavity, resulting in a decrease in the enantioselectivity. The results obtained in this study indicate that at low TEAA concentrations the separation efficiency is the limiting factor for the resolution. An increase in TEAA concentration increases the column effiency, thus increasing the resolution. As the TEAA concentration increases in the mobile phase, the enantioselectivity becomes the limiting factor, thus the resolution decreases with the increasing TEAA concentration. When the TEAA concentration increases to 1.5%, no enantiomeric separation can be observed for most of these DNP-amino acids.

4.3.5 Effect of pH

The influence of pH on the retention and resolution of DNP-DL-amino

acids was investigated by changing the pH of the mobile phase from 4.5 to 7.0 using 0.5% TEAA buffer. Some typical plots of the capacity factor and resolution versus pH values are shown in Fig. 4.6. The retention time of all the DNP-amino acids decreased with increasing pH. This fact can be explained by considering the effect of pH on the form of DNP-amino acids and on the bonding strength of the carboxylic acid functional group to the hydroxyl group of ß-cyclodextrin.

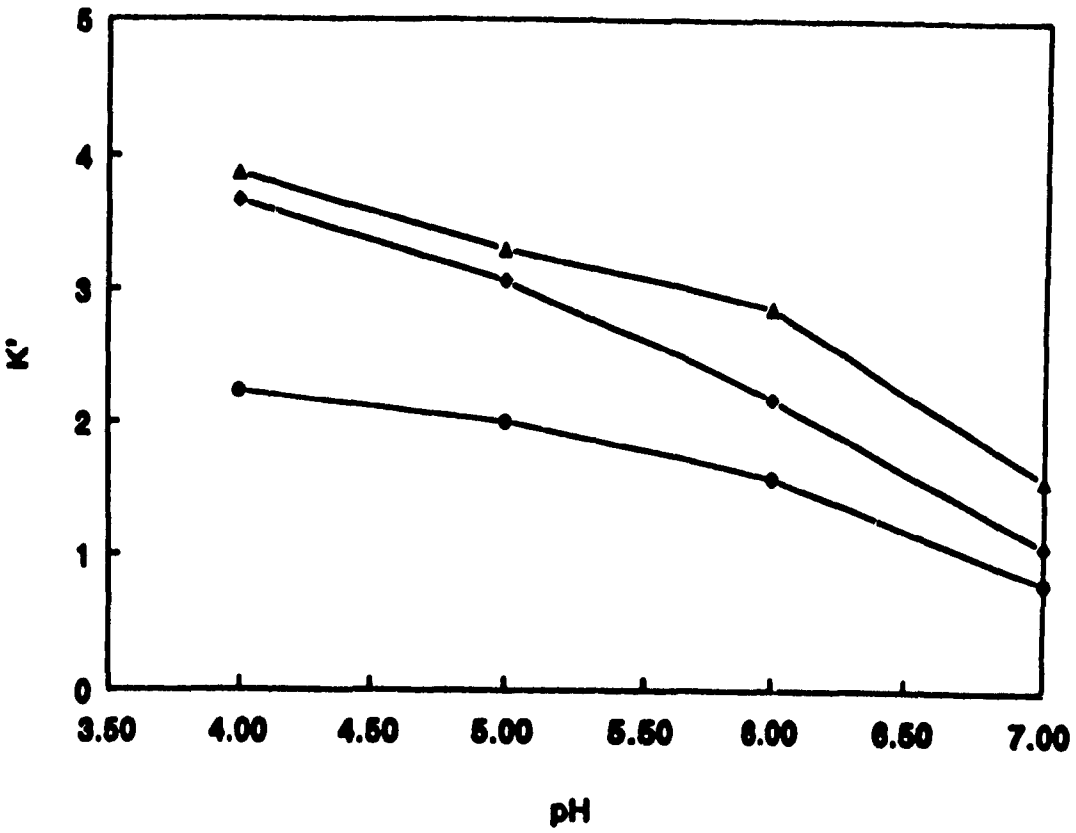


Figure 4.6. Effect of pH on the retention of (▲) DNP-DL-ethionine; (●) DNP-DNP-methionine; (◆) DNP-DL-norleucine. Column, Cyclobond I (250 x 4.6 mm); mobile phase, 30:70 methanol/TEAA buffer (0.5% TEAA); temperature, 20°C; flow rate, 1 mL/min. k' is the capacity factor of the first eluted enantiomer.

By N-substitution, the DNP-amino acids have lost the dipolar ion character of the parent amino acids, and hence can be considered as carboxylic acids with a -NH(DNP) substituent mostly at the α-position. Since the NH(DNP) group is a strong electron-withdrawing group, the pK_a values of the DNP-amino acids should be much lower than that of the corresponding carboxylic acids, even much lower than the pK_a value (3.71) of N-acetylglycine ($CH_3CONHCH_2COOH$) [16]. Therefore, it can be expected that the DNP-amino acids exist mainly in the form of anions in the pH range of 4.5 to 7.0. Thus, the strength of the inclusion complexation between the DNP substituent and the cavity of ß-cyclodextrin should not be affected by changing pH.

However, it has been reported that the OH^- ion had a high hydrogen bonding ability to the hydroxyl groups of ROH molecules [17, 18]. The existence of OH^- in the mobile phase will compete with the carboxylate group of DNP-amino acids to interact with the hydroxyl groups of the cyclodextrin. An increase in OH^- concentration in the mobile phase, caused by the increasing pH, weakens the bonding strength between the carboxylate group of the solute and the hydroxyl group of ß-cyclodextrin. Thus, the overall interaction between the solute and ß-cyclodextrin is weakened. Therefore, a decrease in retention time with an increase of pH is observed.

Fig. 4.7 shows that the optical resolution are relatively little affected by changing pH. This suggests that the interaction between the polar groups of solute and the hydroxyl groups of cyclodextrin may not be the main factor for the chiral recognition.

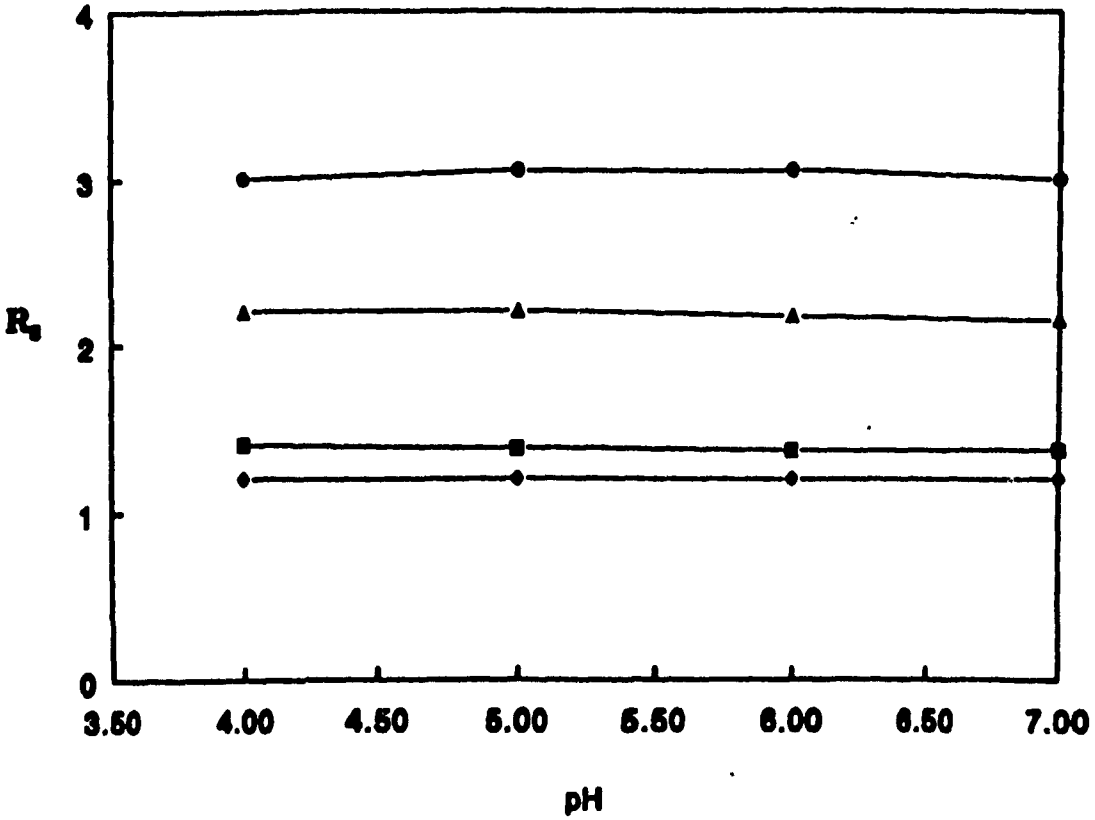


Figure 4.7. Effect of pH on the resolution of (▲) DNP-DL-ethionine; (●) DNP-DL-α-amino-n-caprylic acid; (■) DNP-DL-norleucine; (◆) DNP-DL-methionine. Column, Cyclobond I (250 x 4.6 mm); mobile phase, 30:70 methanol/TEAA buffer (0.5% TEAA); temperature, 20°C; flow rate, 1 mL/min.

4.4 CONCLUSIONS

It has been demonstrated that the ß-cyclodextrin-bonded phase column exhibits a high enantioselectivity for the DNP-amino acid derivatives. This provides a new approach for the enantiomeric separation of racemic amino

acids. This approach also provides a new method for the stereochemical investigation of proteins and peptides. The peptides or proteins react with fluoro-2,4-dinitrobenzene in alkaline solution to give DNP derivatives [19]. Upon hydrolysis, the peptide chain is broken into free amino acids but the original N-terminal amino acid remains largely in the form of its DNP derivatives which can be resolved and identified using the approach described here.

The results suggest that the inclusion complex formation between the CD cavity and the DNP substituent and the steric hindrance of the alkyl substituents around the chiral center of the solutes are the most important factors in the chiral recognition.

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Chapter 5

CD, UV and ^{1}H NMR STUDIES OF THE CHIRAL RECOGNITION MECHANISM OF ß-CYCLODEXTRIN

5.1. INTRODUCTION

In recent years, cyclodextrins have received a great deal of attention as chiral mobile phase additives [1] or as chiral stationary phases [2,3] for direct enantiomeric separations. The tack of most investigators has been to expand their chiral separation spectrum as well as to improve the enantioselectivity by chemical modifications. Although a number of empirical and theoretical studies about the chiral recognition mechanism have been done, relatively little experimental effort has been focused on the explanation of the nature of the chiral discrimination interaction and the chirality forces responsible for the different retention of enantiomers. Enantioselective inclusion complex formation is considered to provide the essential discrimination in cyclodextrin stationary phases. However, very little direct experimental evidence can be found in the literature.

As mentioned in Chapter 1, cyclodextrins are capable of forming

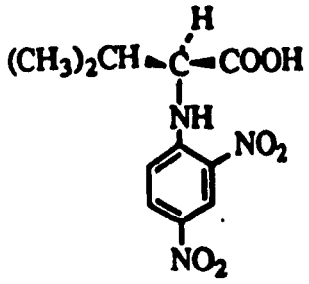
complexes in aqueous solution with a variety of molecular species [4]. The complexes are usually regarded as inclusion compounds in which hydrogen bonding [5,6], and van der Waals forces [7,8] are the main binding forces. Inclusion complex formation has been studied by a variety of methods, such as electron-spin resonance (ESR) spectroscopy [9,10], proton nuclear magnetic resonance (^{1}H NMR) spectroscopy [11-13], ultraviolet (UV) and visible spectroscopy [14-16], circular dichroism (CD) spectroscopy or optical rotatory dispersion (ORD) [17-20], Raman spectroscopy [21], fluorescence spectroscopy [22,23], X-ray analysis [24-27], potentiometry [28], positron annihilation [29] and thermoanalytical methods [30]. Among these methods, ^{1}H NMR spectroscopy, UV-visible spectroscopy and CD method are considered to be the most popular.

Since ^{1}H NMR spectroscopy was first used for the study of adduct formation in aqueous solution, NMR studies of cyclodextrin complexes with aromatic compounds have been made by several researchers [11,31, 32]. In the structure of ß-cyclodextrin, only the 3' and 5' protons are located inside the cavity. The 3' protons form a ring near the larger opening of the cavity, while the 5' protons are near the smaller opening. The 6' protons are located on the upper surface and directed inwards in the gg conformation. All other protons are located on the exterior of the cavity. It has been found that when an aromatic moiety of the guest molecule is included in the cavity of ß-cyclodextrin, protons (3' and 5') located within the cavity are susceptible to anisotropic shielding of the aromatic moiety, while protons located on the exterior of the ß-cyclodextrin cavity, such as 1', 2', and 4', are relatively unaffected. The 6' protons are also affected, though to a lesser extent. Alternatively, if association takes place at the exterior of the cavity, the protons on the exterior of the cavity will be significantly changed. It is now believed that ^{1}H NMR spectra can give the most direct evidence for the inclusion complex formation between guest molecules and the cyclodextrin cavity in aqueous solution.

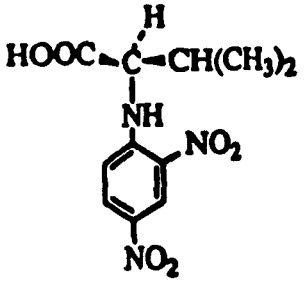
It is known that the formation of inclusion complexes with cyclodextrins leads to the changes of UV-visible spectra of a variety of organic molecules ranging from organic dyes [33] such as marine blue, methyl orange, and crystal violet to aromatic compounds such as phenol, naphthol, and substituted phenolic compounds. The stoichiometries of the inclusion complexes can be found from the UV-visible spectra in spectrophotometric titration, and the formation constants of inclusion complexes can be obtained from the absorption changes. Circular dichroic (CD) spectroscopy of active compounds is also a powerful tool for the study of the three-dimensional structures of organic molecules [34,35]. This technique provides information on the absolute configuration, conformation, and reaction mechanism, etc. It is shown that circular dichroic spectroscopy is also a powerful method for investigating the cyclodextrin-guest interaction, since the guest chromophore perturbed by the cyclodextrin molecule produces the induced circular dichroism spectrum [19]. Both formation constants of inclusion complexes and the geometry of the host-guest interactions can be evaluated from the induced CD spectra.

In this chapter, I report my efforts to employ high resolution NMR, UV-visible, and circular dichroism (CD) spectroscopies as probes for the study of the host-guest interactions which lead to chiral recognition using DNP-amino acids as model compounds. The structures of these model solutes are shown in Figure 5.1.

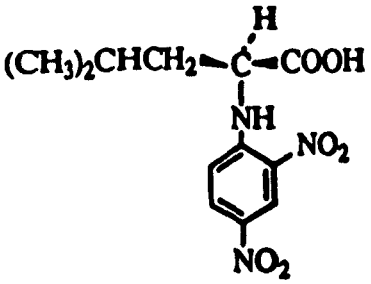
The aim of this work is to show that these DNP-amino acids actually form enantioselective inclusion complexes with ß-cyclodextrin in the aqueous solution and to characterize the chiral separation mechanism by UV-visible, CD and ^{1}H NMR spectrometric measurements. Furthermore, I desire to elucidate the mode of inclusion and the structure of the complex from the induced CD bands and NMR spectra. The induced CD bands, UV absorption changes and NMR chemical shift changes were quantitatively investigated to obtain the dissociation constants.



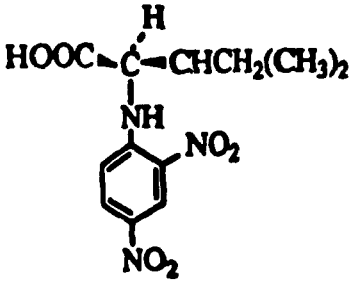
DNP-L-Valine



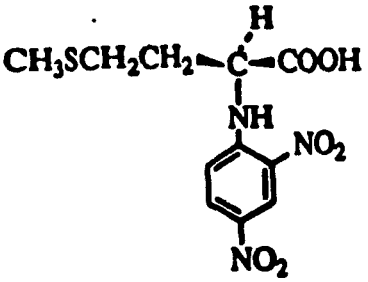
DNP-D-Valine



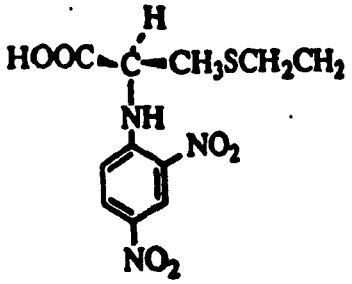
DNP-L-Leucine



DNP-D-Leucine



DNP-L-Methionine



DNP-D-Methionine

Figure 5.1. The structures of the DNP-amino acids used as model solutes.

5.2. EXPERIMENTAL

5.2.1. Materials

ß-Cyclodextrin was purchased from Aldrich Chemical Co. and purified by recrystallization once from propanol followed by recrystallization once from water and drying at 80°C with P_2O_5 *in vacuo* for 12 hrs. The specific rotatory power of ß-cyclodextrin was $[\alpha]^{25}_D$=162.0±0.5. Fluoro-2,4-dinitrobenzene (FDNB), DNP-L-valine, DNP-L-leucine, and other D- and L-amino acids were from Sigma Chemical Co. DNP-D-Valine, DNP-D-leucine and D- and L- DNP-methionine were prepared by modifying the method described by Schroeder and Legette [36].

5.2.2 CD, UV and NMR measurements

CD measurements were carried out in a 0.1 M sodium phosphate buffer, pH 6.0, on a Jasco-500C Spectropolarimeter (Japan Spectroscopic CD, Japan) with a JASCO DP-J800/30 data processor. A 1-cm-thick quartz cell was used to hold the sample solution. The CD spectra in the range of 600 nm to 185 nm were measured at the following set of operating parameters: sampling wavelength, 1 nm; scan speed, 100 nm/min; time constant, 2 seconds; and temperature, 21 ± 0.5°C. The recording of each spectrum was repeated four times. and the averaged spectra were obtained on the data processor.

NMR spectra were measured by a XL-200 spectrometer (Varian Canada Ltd., Montreal) at ambient probe temperature of 20 ± 1°C. The sample was prepared in a 0.05 M phosphorate buffer solution. Since the solubilities of DNP-amino acids at pH = 6.0 are not high enough for NMR measurement, a buffer solution of pH 11.0 was used. The buffer solution was prepared with

anhydrous Na_3PO_4 in D_2O and the pD was adjusted with deuterium chloride acid. The pD value was obtained by adding 0.4 to the pH meter reading [37], using a glass electrode which had been calibrated with pH 7.00 and pH 10.00 buffers in water and then rinsed with D_2O. No internal ^1H NMR reference was added since the possibility of reference binding to the ß-cyclodextrin could not be excluded. A solvent line (4.63 of D_2O) was used as the reference.

The UV-visible spectra were measured on a DMS-300 UV-Visible spectrophotometer (Varian Canada, Montreal) in a 0.1 M sodium phosphate buffer solution of pH 6.0. The sample compartment contained a 1-cm-thick quartz cell with combined ß-cyclodextrin and DNP-amino acid solution while the reference compartment contained the same concentration of ß-cyclodextrin as the sample cell but with no DNP-amino acid.

5.2.3. Determination of dissociation constants by UV-visible spectra.

The dissociation constants, K_d, for ß-cyclodextrin-DNP-amino acid complexes were measured according to the conventional Scott's equation (1) [38], (see Appendix A).

$$[H_0]\bullet[G_0]/\Delta Abs = K_d/\Delta\varepsilon + ([H_0 + [G_0])/\Delta\varepsilon \qquad (1)$$

where $[G_0]$ is total concentration of DNP-amino acids, $[H_0]$ is the total concentration of ß-cyclodextrin, $\Delta\varepsilon$ is the difference of the molar absorptivities for free and complexed DNP amino acids, and ΔAbs is the change in absorbance by the addition of ß-cyclodextrin. The data were treated by plotting $[H_0]\bullet[G_0]/\Delta Abs$ vs $([H_0] + [G_0])$, providing a slope of $1/\Delta\varepsilon$ and a intercept of $K_d/\Delta\varepsilon$. In order to meet the requirement for a linear plot, i.e $[H_0]\bullet[G_0] >> C^2$ (C is the concentration of host-guest complex), the concentration of DNP-amino

acid is held at least ten times lower than the lowest ß-cyclodextrin concentration.

5.2.4. Determination of dissociation constants by CD spectra

The changes of the ellipticities of DNP-amino acids were measured as a function of ß-cyclodextrin concentrations. The data were also handled according to a modified Scott's equation (2),

$$[H_0]\bullet[G_0]/\Delta\theta = K_d/\Delta[\theta] + ([H_0 + [G_0])/\Delta[\theta] \qquad (2)$$

where $\Delta\theta$ is the observed change of ellipticity of the guest molecule, and $\Delta[\theta]$ is the difference of molecular ellipticity of guest before and after the inclusion complexation. Here, $[H_0]\bullet[G_0]/\Delta\theta$ was plotted against $([H_0] + [G_0])$. The K_d was obtained by dividing the intercept by the slope. In these measurements, the concentration of DNP-amino acids was held constant at 7.0×10^{-5} M while the concentrations of ß-cyclodextrin were changed from 0.001 to 0.014 M.

5.2.5. Determination of dissociation constant by NMR

^{1}H Fourier transform NMR spectra (200 MHz) were measured on a LX-200 spectrometer. The chemical shifts of the 3' protons of ß-cyclodextrin were measured as a function of the concentrations of DNP-amino acids. The concentration of ß-cyclodextrin was held constant at 0.001 M and the concentrations of DNP-amino acid were varied between 0.01 to 0.02 M. The data were treated according to Bergeron and Channing's equation (3) [39], (see Appendix B).

$$[G_0]/\Delta\delta = K_d/Q + ([G_0] + [H_0])/Q \qquad (3)$$

where $[G_0]$ is the concentration of DNP amino acids, $[H_0]$ is the concentration of ß-cyclodextrin, $\Delta\delta$ is the change of chemical shift, and $Q = (\delta_C - \delta_H)$. $[G_0]/\Delta\delta$ was plotted against $([G_0] + [H_0])$. The dissociation constants were obtained by dividing the intercept by the slope.

5.3. RESULTS AND DISCUSSION

5.3.1. UV-Visible Studies

Figure 5.2 shows the UV-visible spectra of all the DNP-amino acids in a 0.10 M sodium phosphate buffer solution in the absence of ß-cyclodextrin. The UV-visible spectra of all the DNP-amino acids investigated show two absorption maxima at about 361 nm and 263 nm, and both the L-enantiomer and the D-enantiomer of one DNP-amino acid have the same spectra in the absence of ß-cyclodextrin.

The effect of ß-cyclodextrin on the UV-visible spectra of DNP-amino acids was investigated by holding the concentrations of DNP-amino acids constant at 1.0×10^{-4} M and varying the concentrations of ß-cyclodextrin from 0.001 M to 0.015 M. As a typical example of the observed spectral change with the addition of ß-cyclodextrin, Figure 5.3 shows the UV-visible spectra of DNP-L-valine at various ß-cyclodextrin concentrations. In the presence of ß-cyclodextrin the absorption maximum at 361 nm was shifted to longer wavelengths (3-5 nm) along with some lowering of the absorption intensity. The well-defined isobestic points at about 330 nm and 385 nm indicate a 1:1 complex. Similar UV-visible spectra were also observed for the DNP-D-valine

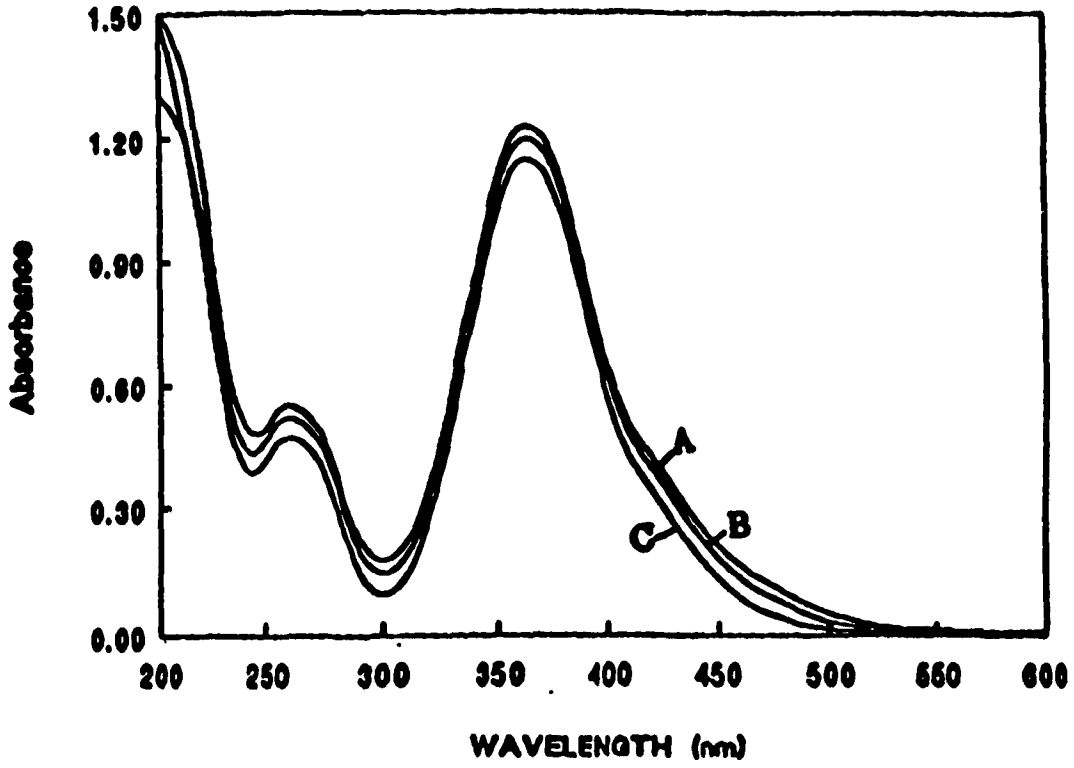


Figure 5.2. UV-Visible spectra of the DNP-amino acids in the solvent of 0.10 M phosphate buffer, pH = 6.0. (A) DNP-leucine, (B) DNP-valine, and (C) DNP-methionine. The concentration of DNP amino acids is 1.0×10^{-4} M.

and other DNP amino acids. However, the changes of absorption intensity at the absorption maximum on adding ß-cyclodextrin are different for L- and D-enantiomers. A plot of the data in the form of $[H_o]\cdot[G_o]/\Delta abs$ vs $([H_o] + [G_o])$ showed excellent straight line fits (see Figure 5.4), indicating an H + G = HG equilibrium model.

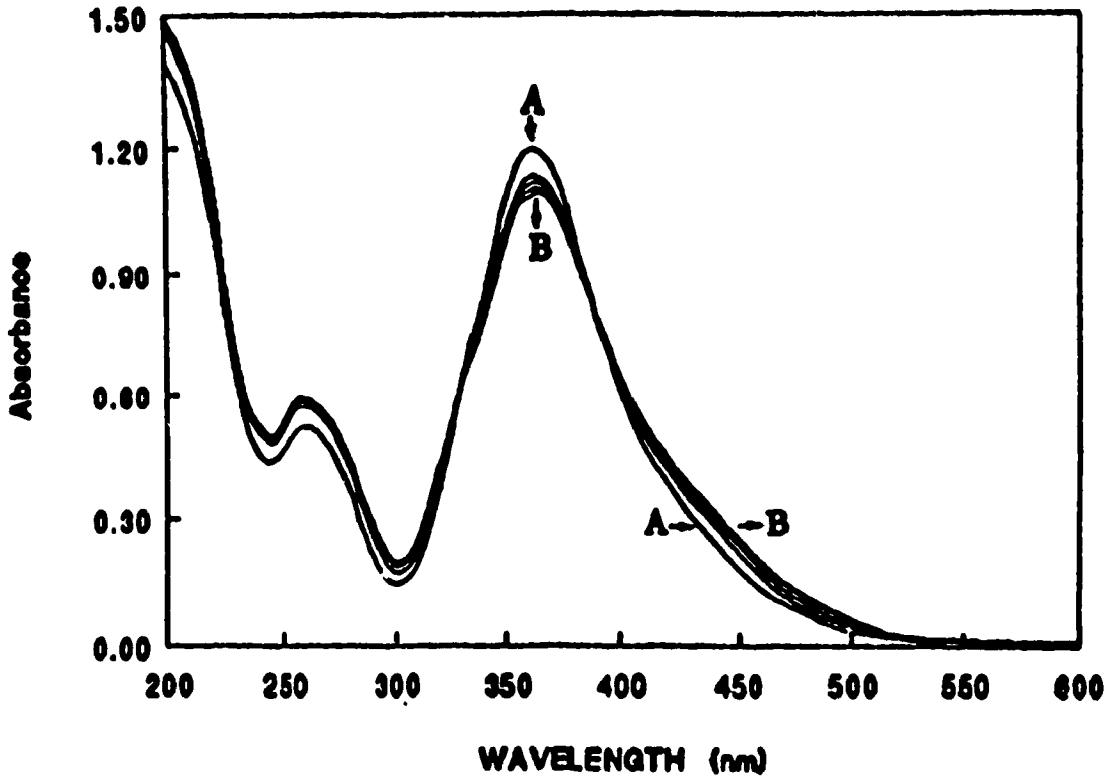


Figure 5.3. UV-visible spectra of DNP-L-valine at varying ß-cyclodextrin concentrations: 0, 1.0 x 10^{-3}, 5.0 x 10^{-3}, 1.0 x 10^{-2}, and 1.5 x 10^{-2} M, read from A to B. The concentration of DNP-L-valine is 1.0 x 10^{-4} M, and the measurements were carried out in a 0.10 M phosphate buffer (pH = 6.0).

Table 5.1 summarizes the dissociation constants for all the L- and D-DNP amino acids along with the capacity factors obtained on a ß-cyclodextrin bonded stationary phase. As is seen in Table 5.1, all the L-enantiomers have longer retention times on the ß-cyclodextrin bonded phase column and show larger formation constants than the corresponding D-enantiomers.

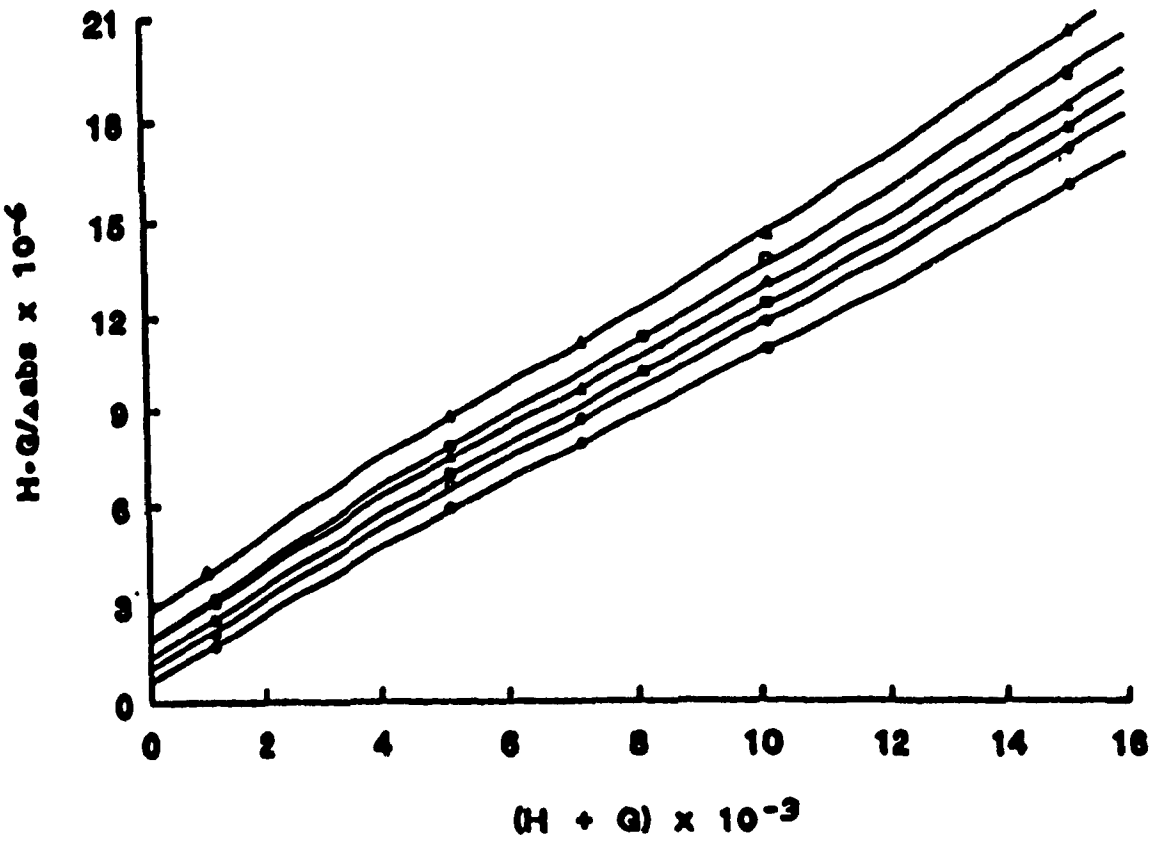


Figure 5.4. Scott's plots for the interactions between ß-cyclodextrin and (▲) DNP-L-valine, (△) DNP-D-valine, (•) DNP-L-leucine, (○) DNP-D-leucine, (■) DNP-L-methionine, and (□) DNP-D-methionine.

Table 5.1. Dissociation Constants for Inclusion Complexes of ß-Cyclodextrin : DNP-amino acids

Guest Molecules	K_a (UV,M)	K_a(CD,M)	K_a(NMR,M)	T_R (min)
DNP-L-valine	$1.78\pm0.10 \times 10^{-3}$	$2.83\pm0.05 \times 10^{-3}$	2.85×10^{-3}	23.8
DNP-D-valine	$2.36\pm0.17 \times 10^{-3}$	$3.26\pm0.05 \times 10^{-3}$	3.38×10^{-3}	20.6
DNP-L-leucine	$7.11\pm0.41 \times 10^{-4}$	$1.66\pm0.20 \times 10^{-3}$	1.52×10^{-3}	38.2
DNP-D-leucine	$1.15\pm0.21 \times 10^{-3}$	$2.15\pm0.25 \times 10^{-3}$	2.43×10^{-3}	28.6
DNP-L-methionine	$1.38\pm0.19 \times 10^{-3}$	$1.32\pm0.07 \times 10^{-3}$	2.47×10^{-3}	25.8
DNP-D-methionine	$1.78\pm0.25 \times 10^{-3}$	$2.16\pm0.41 \times 10^{-3}$	2.96×10^{-3}	23.1

5.3.2. Circular Dichroism Studies

5.3.2.1. Comparison of the CD spectra of DNP amino acids with that of original amino acids.

CD spectra of D- and L- DNP valine in the absence of ß-cyclodextrin are shown in Figure 5.5. The DNP-L-valine gives a negative CD band centred at 225 nm, while one positive CD peak appears at 410 nm. In contrast, the DNP-D-valine has a positive CD peak at 225 nm and a weaker negative peak at

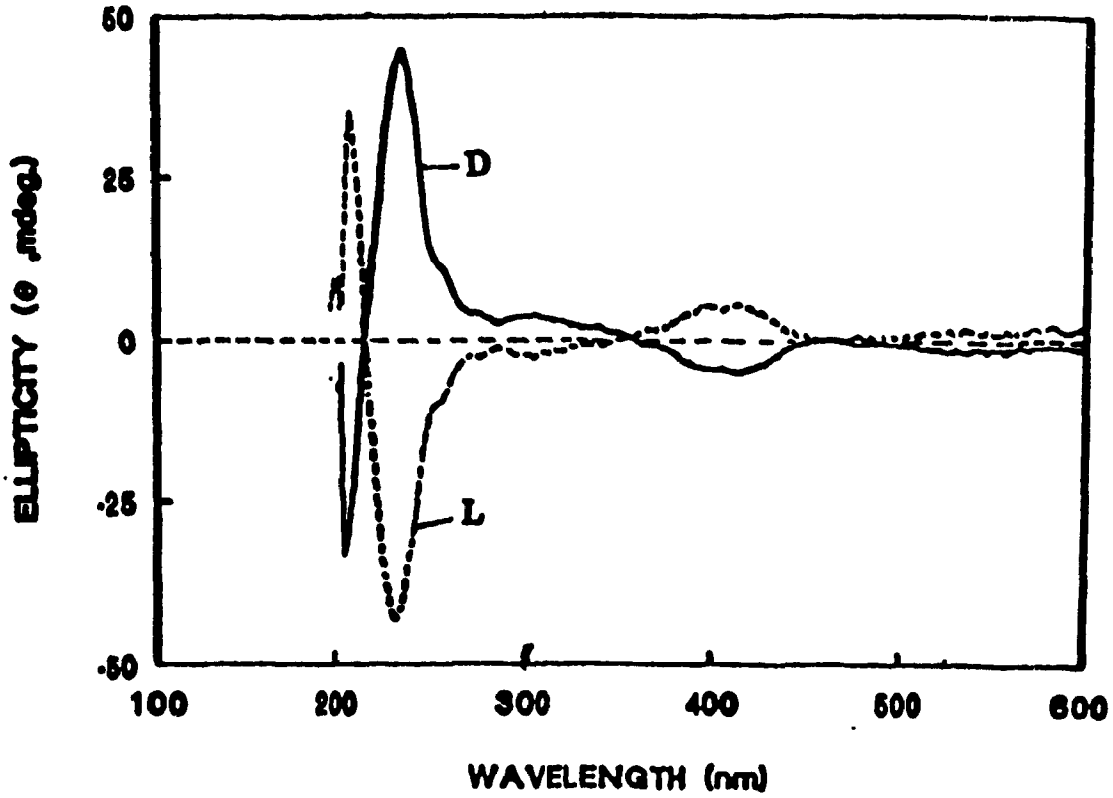


Figure 5.5. CD spectra of DNP-L-valine (---) and DNP-D-valine (---) in the 0.10 M phosphate buffer (pH = 6.0). The concentration of both enantiomers are 7.0×10^{-5} M.

410 nm. It appears that the peak at 225 nm results from the n-π^* Cotton effect of the carboxyl group while the CD peak at 410 nm is associated with the π-π^* transition of the dinitrophenyl group. Similar CD spectra were also observed for other D- and L- DNP amino acids. Compared with the CD spectra of the original amino acids, the observed molecular ellipticity of the DNP amino acids at 225 nm is about three times as large as that of the original amino acids. The most remarkable change is the sign of the CD band at about 225 nm. It has been reported that all L-α-amino acids, except the cyclic amino acid proline, give positive Cotton effect curves with CD peaks in the wavelength range from 213 nm to 228 nm [40]. The negative CD peaks for L- DNP amino acids at 225 nm indicate that the DNP derivatization of amino acids changes the sign of the Cotton effect associated with the carboxyl group. This discovery helped me to discover and correct the mistake I made in determining the elution order of DNP-DL-amino acids on a ß-cyclodextrin column [41]. Now, it is found that the first eluted enantiomer possessing a positive CD peak at 225 nm is the DNP-D-amino acid, rather than the DNP-L-amino acids as earlier reported.

The changed sign of the Cotton effect for DNP amino acids can be easily explained by considering the Sector Rule derived from the Octant Rule by Klyne and Scopes [42]. In the Sector Rule, each carbon-oxygen bond of the carboxyl group is considered in turn as a double bond, and the signs of the contributions made by substituents are allocated according to the ketone Octant Rule [43, 44]. The diagrams I and II in Figure 5.6 show the two patterns for the upper octants while the signs of the lower sectors are opposite to those of the upper sectors. When these two diagrams are superimposed, the pattern shown in diagram III is produced, giving a strong positive contribution in the back upper right sector and a negative contribution in the back upper left sector. The explanation of the signs of the Cotton effect for the original and the DNP amino acids may be illustrated by considering a typical example shown in Figure 5.7. As shown in diagram I of Figure 5.7, for the L-amino acid configuration, the alkyl group is in the positive upper right sector while

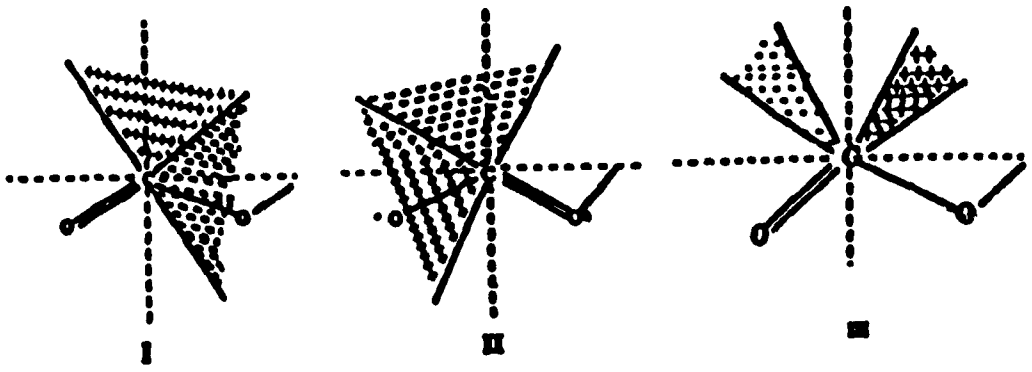


Figure 5.6. The Sector Rule [35].

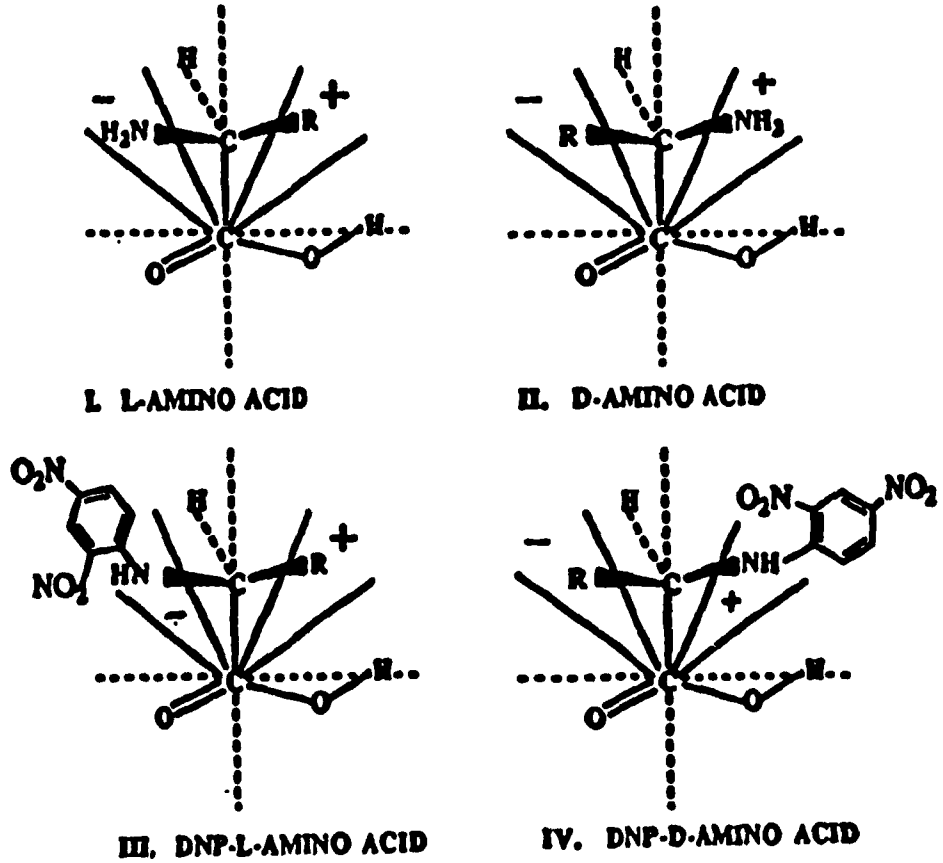


Figure 5.7. Sector projections of D and L amino acid and their DNP derivatives.

the amine group is located in the negative upper left sector. The positive Cotton effect of carboxyl group at about 225 nm for L-amino acid is mainly contributed by the alkyl group. For the D-amino acid configuration as shown in diagram II of Figure 5.7, the alkyl group is in the negative upper left sector while the amine group is located in the positive upper right sector. Therefore, a negative Cotton effect is observed. However, after the substitution of dinitrophenyl group for hydrogen on the amine group, the DNP group becomes the main contributor to the Cotton effect of the carboxyl chromophore. The DNP group is located in the negative upper-left sector for DNP-L-amino acids and in the positive upper-right sector for DNP-D-amino acids. Thus, the situation is reversed. A negative Cotton effect is observed at 225 nm for the DNP-L-amino acids, and a positive Cotton effect is observed for the DNP-D-amino acid.

5.3.2.2. CD spectra of amino acids in the presence of ß-cyclodextrin

Figure 5.8 shows the CD spectra of DNP L-valine at varying ß-cyclodextrin concentrations. In this case, the concentration of DNP L-valine was held constant at 7.0×10^{-5} M, and the CD spectra intensity were measured at ß-cyclodextrin concentrations of 1.0×10^{-3}, 5.0×10^{-3} and 9.0×10^{-3} M. An increase in ß-cyclodextrin concentration lowers the intensity of the negative CD peak at 225 nm. In the presence of ß-cyclodextrin, a broadened CD band is observed in the region of 270 nm to 430 nm. Compared to the CD spectra of DNP-L-amino acid in the absence of ß-cyclodextrin, we found that the broadened CD band consists of two components, the intrinsic part and the induced part, which is superimposed on the intrinsic part.

The CD spectra of the DNP-D-valine at different ß-cyclodextrin concentrations are shown in Figure 5.9. The intensity of the CD peak at 225 nm becomes lower with increasing ß-cyclodextrin concentrations. However, unlike the CD spectra of DNP-L-valine, only an independent induced CD band is observed in the range of 290 nm to 370 nm region. This induced positive

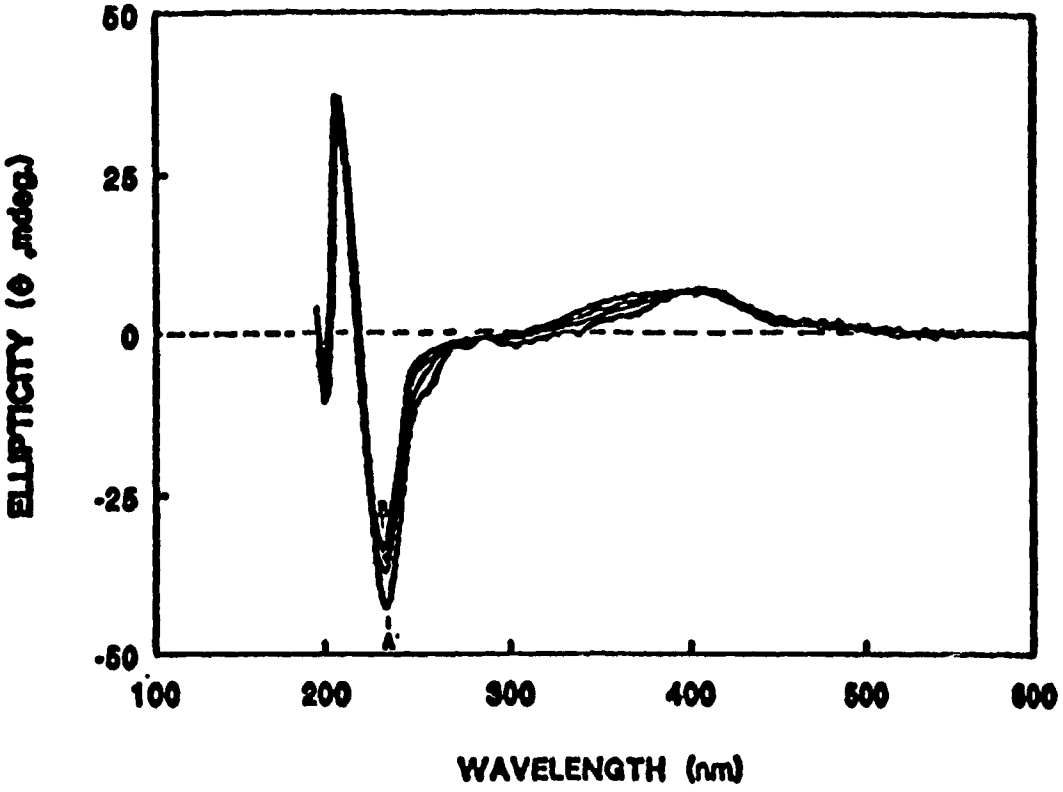


Figure 5.8. CD spectra of DNP-L-valine at varying ß-cyclodextrin concentrations: 0, 1.0 x 10^{-3}, 5.0 x 10^{-3}, and 9.0 x 10^{-3} M, read from A to B. The concentration of DNP-L-valine is 7.0 x 10^{-5} M. The 0.10 M phosphate buffer solution (pH = 6.0) was used as solvent.

band is centred at about 350 nm with an intensity higher than the induced CD peak of DNP-L-valine.

The difference in the shape and intensity of the induced CD spectra of DNP-L-valine and DNP-D-valine in the presence of ß-cyclodextrin is mainly ascribed to the different orientation and/or disposition of the D- and L-

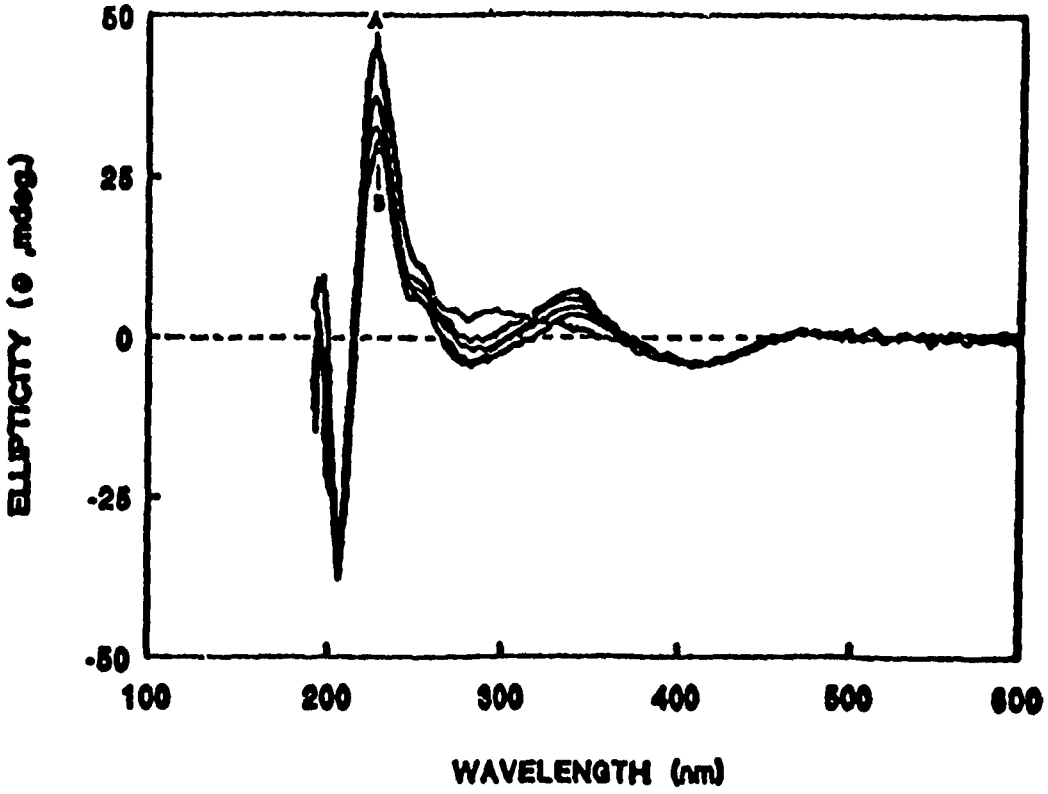


Figure 5.9. CD spectra of DNP-D-valine at varying ß-cyclodextrin concentrations: 0, 5.0×10^{-4}, 1.0×10^{-3}, 5.0×10^{-3}, and 9.0×10^{-3} M, read from A to B. The concentration of DNP-D-valine is 7.0×10^{-5} M; solvent, 0.10 M phosphate buffer (pH = 6.0).

enantiomers (or the DNP groups) in the ß-cyclodextrin cavity. The intensity of induced CD band by ß-cyclodextrin can be interpreted in terms of the Kirkwood-Tinoco coupled oscillator model [20, 45]:

$$R = A(1 + 3\cos 2\theta)\mu^2$$

where, R is the rotation strength or CD intensity, A is a constant dependent only on the wavelength, and θ is the angle made by the direction of electric dipole moment (μ) of the guest molecule and the symmetry axis of the ß-cyclodextrin. The rotation strength is independent of the rotation of the guest molecule around the symmetry axis of ß-cyclodextrin, but sensitive to the angle θ. The sign of the induced CD band is determined only by the relative orientation of the dipole moment in the ß-cyclodextrin cavity. The electric dipole moment parallel to the symmetry axis of ß-cyclodextrin gives a positive CD band while the perpendicularly polarized moment produced the negative CD band. The observed positive CD bands induced by ß-cyclodextrin indicate that the electric dipole moment in parallel with the long axis of the 2,4-dinitrophenyl group is parallel to the symmetry axis of ß-cyclodextrin. It appears that axial inclusion complexes, as shown in Figure 5.10, are formed between D-, L- DNP-amino acids and ß-cyclodextrin cavity. The lower intensity of the induced CD band for DNP-L-valine indicates that DNP-L-valine has a large tilt angle (θ) against the symmetry axis of ß-cyclodextrin probably due

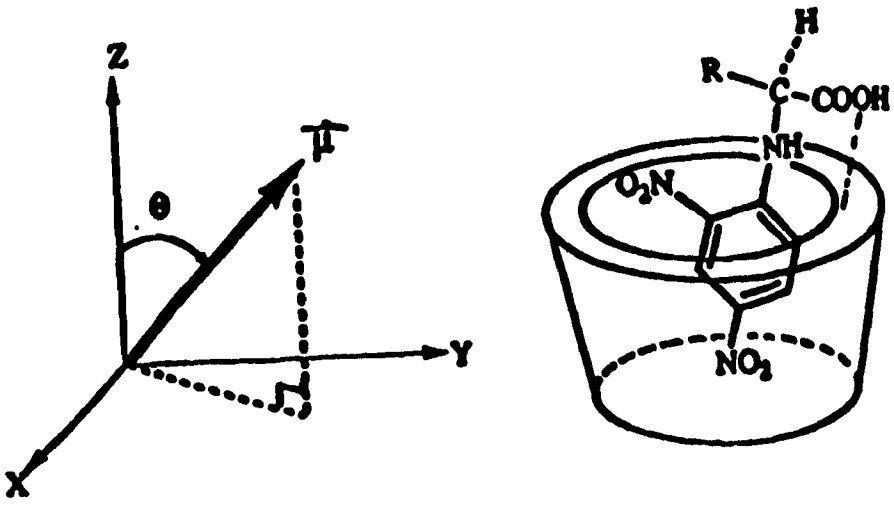


Figure 5.10. (a) The coordinate system of the electric dipole moment; (b) the most likely disposition for ß-cyclodextrin-DNP-amino acid complexes.

to the formation of strong hydrogen-bonding between the carboxyl group of DNP-L-valine and the hydroxyl group on the edge of ß-cyclodextrin. The higher intensity of the induced positive CD band for DNP-D-valine means a smaller tilt angle (θ) between the long axes of the dinitrophenyl group of DNP-D-valine and the symmetric axis of ß-cyclodextrin. The smaller tilt angle may be caused by the steric repulsion between the alkyl group (R) and the hydrophillic surface of ß-cyclodextrin.

The CD spectra shown in Figure 5.8 and Figure 5.9 were also observed for the L- and D-enantiomers of DNP-leucine and DNP-methionine, respectively. In all the cases, it was found that the intensity of the induced CD peaks at 350 nm increased with the increasing ß-cyclodextrin concentrations. However, attempted fit of the ellipticity data for these induced CD peaks to the modified Scott's equation failed. No straight line can be found for the plots of $[H_0][G_0]/\Delta\theta$ versus $([H_0] + [G_0])$ for all the DNP-amino acids studied. The intensity of the intrinsic CD peaks at 225 nm decreased with increasing ß-cyclodextrin concentrations. The ellipticity changes when plotted according to the modified Scott's equation gave straight lines with correlation coefficients larger than 0.9950 for all the D- and L-enantiomers of DNP-amino acids. The dissociation constants obtained are also listed in Table 5.1. These dissociation constants reflect, in some extent, the interactions between the carboxyl group of the DNP-amino acids and the exterior surface of ß-cyclodextrin.

5.3.3. NMR Studies

The ^1H NMR spectrum of free ß-cyclodextrin in D_2O is shown in Figure 5.11-A. The spectrum of free ß-cyclodextrin was initially assigned by Demarco and Thakker [46]. The assignments were confirmed in the present work by

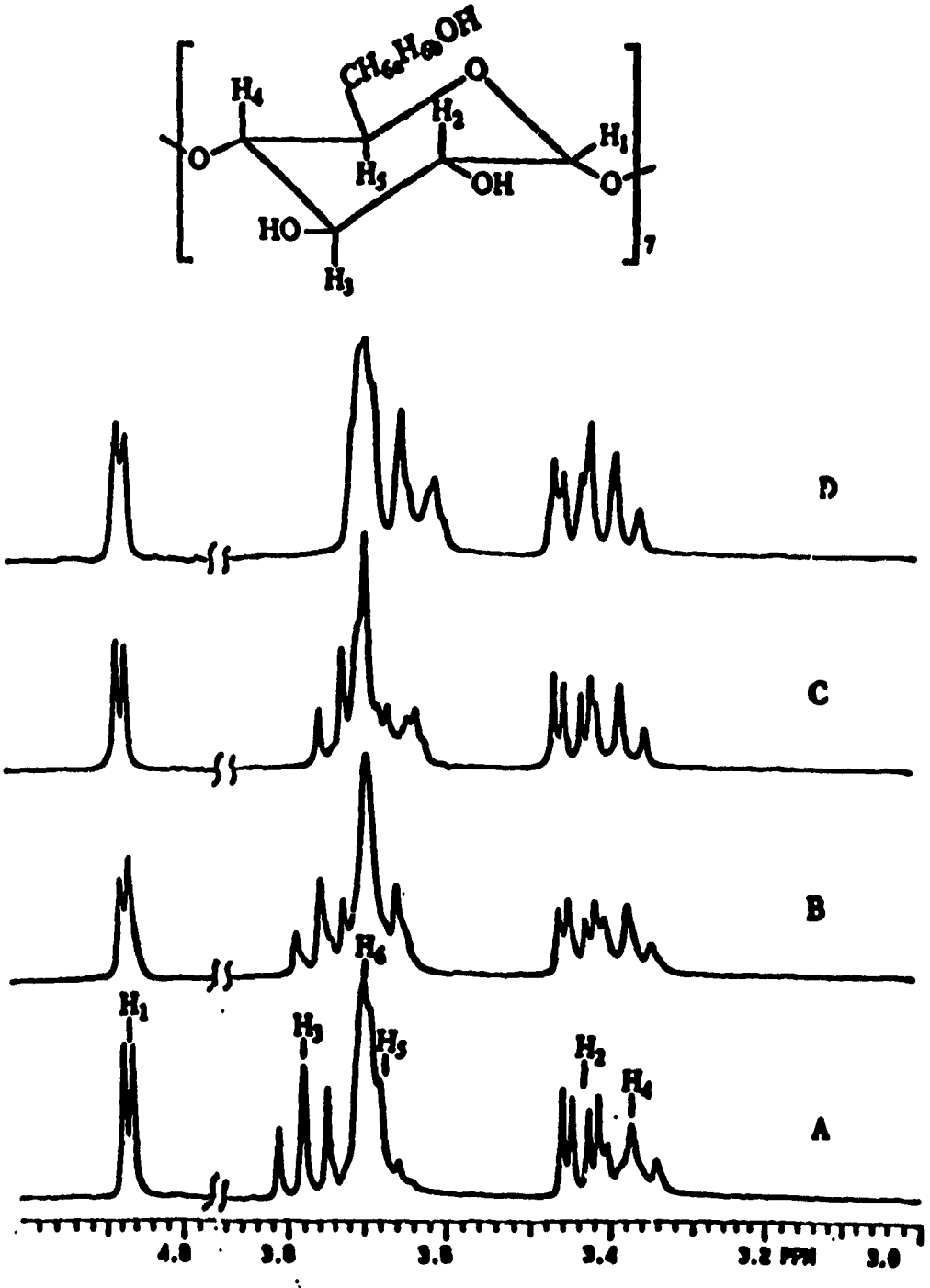


Figure 5.11. ^{1}H NMR (200 Hz) of ß-cyclodextrin at different molar ratio of DNP-L-valine to ß-cyclodextrin: (A) 0, (B) 0.5, (C) 1.0, and (D) 2.0. The ß-cyclodextrin concentration is constant at 0.005 M.

decoupling and COSY experiments. The free ß-cyclodextrin resonance positions at 20 ± 1°C relative to solvent line (D_2O, 4.63 ppm) are 1' at 4.925 (doublet), 2' at 3.477 (doublet of doublets), 3' at 3.824 (triplet), 4' at 3.428 (triplet), 5' at 3,664 (doublet of triplets), both 6' protons nearly overlap at 3.728. In D_2O solution only resonances from the non-changing hydrogens attached to the carbons are detected. The resonances for the active 2', 3' and 6' hydroxyl protons were not observed. (We use i' to assign the ß-cyclodextrin protons, and use iH to assign the aromatic protons of DNP-amino acids.)

5.3.3.1. Effect of DNP-amino acid on the ^1H NMR spectra of ß-cyclodextrin.

The effects of DNP-amino acids on the spectrum of ß-cyclodextrin were investigated by holding the concentration of ß-cyclodextrin constant and changing the molar ratios of ß-cyclodextrin to DNP-amino acid from 0 to 20. Figure 5.11-B, C, and D show the effect of DNP-L-valine on the ^1H NMR spectrum of ß-cyclodextrin. As expected, the lower field triplet assigned to 3' proton resonance is progressively shifted to higher field with increasing concentration of DNP-L-valine in the solution. Upfield shift is also observed for the 5' proton resonances which originally superimposed on the 6' proton signals. No significant chemical shift or line-broadening is observed for 2', 4' and 6' protons.

Figure 5.12 shows the plots of chemical shift values for the protons of ß-cyclodextrin as a function of R, the molar ratio of DNP-L-valine to ß-cyclodextrin. The chemical shift of 1' proton is used as the reference for the calculation of $\Delta\delta$ values. As can be seen in Figure 5.12, The $\Delta\delta$ values for 2',4', and 6' protons are constant to within 2 Hz over the range of R values from 0 to 4.0. This means that the relative shift of the exterior protons are unaffected

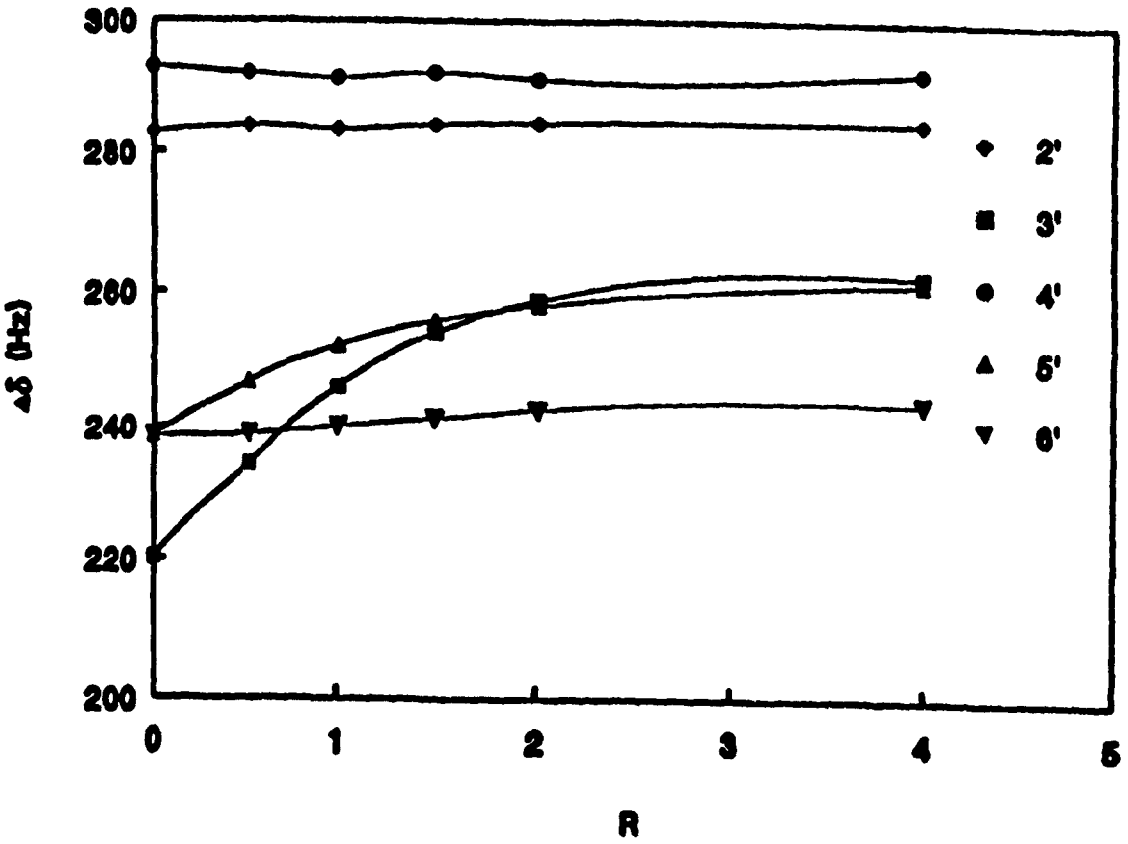


Figure 5.12. The plots of the changes in proton chemical shifts for ß-cyclodextrin in aqueous solution containing different amounts of DNP-L-valine. Δδ is the chemical shift of the protons relative to the 1' proton shift. R is the molar ratio of DNP-L-valine to ß-cyclodextrin.

by the addition of DNP-L-valine. The Δδ value for the resonance of 3' protons increased 38.42 Hz with increasing molar ratio (R) form 0 to 2.0. The effect of DNP-L-valine on the resonance of 5' protons is less substantial. The Δδ value for the resonance of 5' protons only increased 19.33 Hz within the range of molar ratio from 0 to 2.0.

The effect of DNP-D-valine on the 1H NMR spectra of ß-cyclodextrin was

also examined. In Figure 5.13, we plotted the chemical shift ($\Delta\delta$) values for the protons of ß-cyclodextrin against the molar ratio of DNP-D-valine to ß-cyclodextrin. Again, the $\Delta\delta$ values of 2', 4', and 6' are constant over the investigated R range, and upfield shifts were observed for the resonances of 3' and 5' protons. However, the effects of DNP-D-valine on the resonances of 3' and 5' protons are significantly different from the effect of DNP-L-valine. The

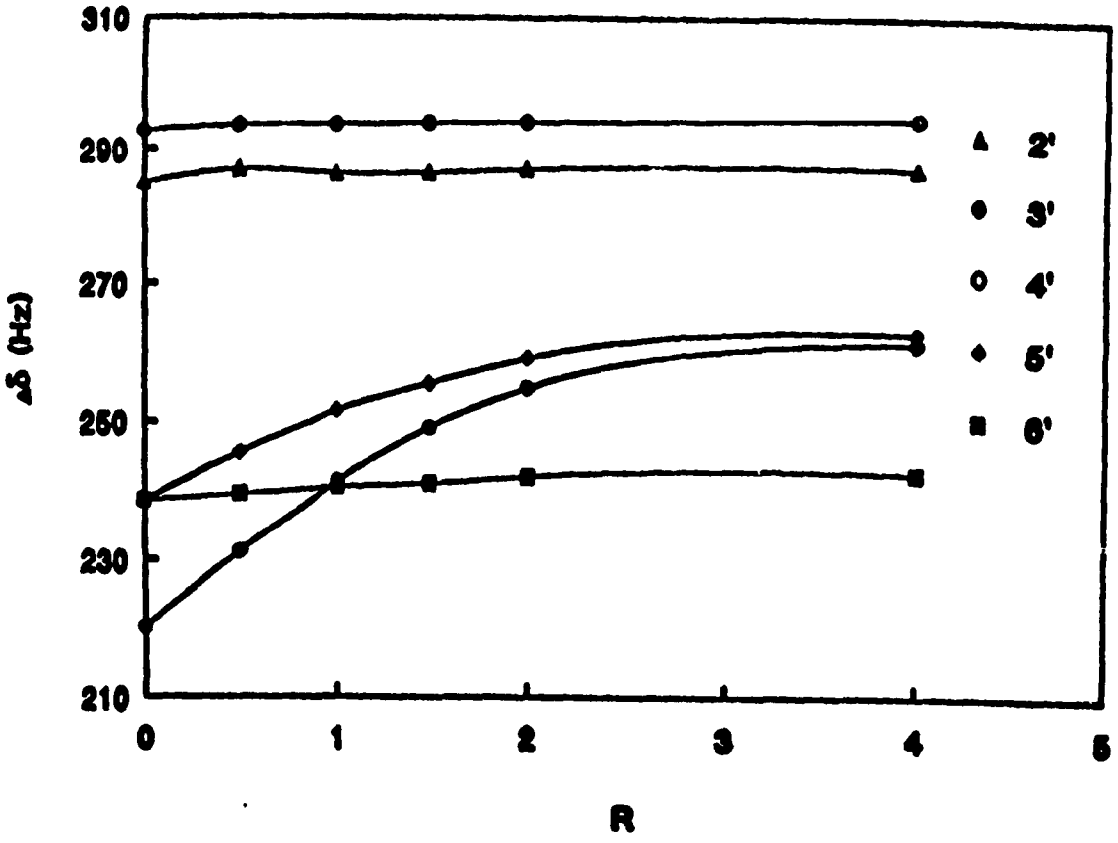


Figure 5.13. The plots of the chemical shift changes for the protons of ß-cyclodextrin against the molar ratio of DNP-D-valine to ß-cyclodextrin.

effect of DNP-D-valine on the resonance of 3' protons is less substantial. Within the range of R from 0 to 2.0, $\Delta\delta$ value for 3' protons increased 33.62 Hz, about 5 Hz lower than the $\Delta\delta$ value observed in the presence of DNP-L-valine. In contrast, the effect of DNP-D-valine on the resonance of 5' protons is more significant than the effect of DNP-L-valine. The $\Delta\delta$ value for 5' protons increased 21.43 HZ with increasing molar ratio from 0 to 2.0, about 2 Hz higher than the $\Delta\delta$ value produced by the effect of DNP-L-valine.

The strong molar ratio dependence of the $\Delta\delta$ values for the 3' and 5' protons is the direct evidence for the inclusion complex formation between the cavity of ß-cyclodextrin and the dinitrophenyl group of DNP-amino acid, since only when the dinitrophenyl group of the DNP-amino acid includes into the ß-cyclodextrin cavity can the strong anistropic shielding of aromatic ring become accessible to the 3' and 5'protons. The $\Delta\delta$ value for the 3' protons which form a ring near the larger opening of the cavity is related to the stability of the inclusion complex [11], while the $\Delta\delta$ value for the 5' protons which form a ring near the smaller opening of the cavity can be taken as an indicator for the penetration depth of the aromatic group of the DNP-amino acid. The larger $\Delta\delta$ value for 3' protons observed in the presence of DNP-L-valine suggests that DNP-L-valine form a more stable inclusion complex with ß-cyclodextrin than its D-enantiomer. The larger $\Delta\delta$ value observed for the 5' protons in the presence of DNP-D-valine indicates that the dinitrophenyl group of DNP-D-valine penetrates into the cavity more deeply than the dinitrophenyl group of DNP-L-valine.

The effects of L- and D-enantiomers of DNP-leucine and DNP-methionine on the spectra of ß-cyclodextrin demonstrate similar results to DNP-L-valine and DNP-D-valine, respectively. Figure 5.14 shows the plots of $\Delta\delta$ values for the resonance of 3' protons versus the molar ratio (R) for all the DNP-amino acids studied. In all the case studied, the effect of L-enantiomer

on the resonances of 3' protons is more pronounced than that of the D-enantiomer. The chemical shift changes of 3' protons with the addition of DNP-amino acids are used to calculate the dissociation constants of the inclusion complex according to Bergeron and Channing's equation. The results are also listed in Table 5.1.

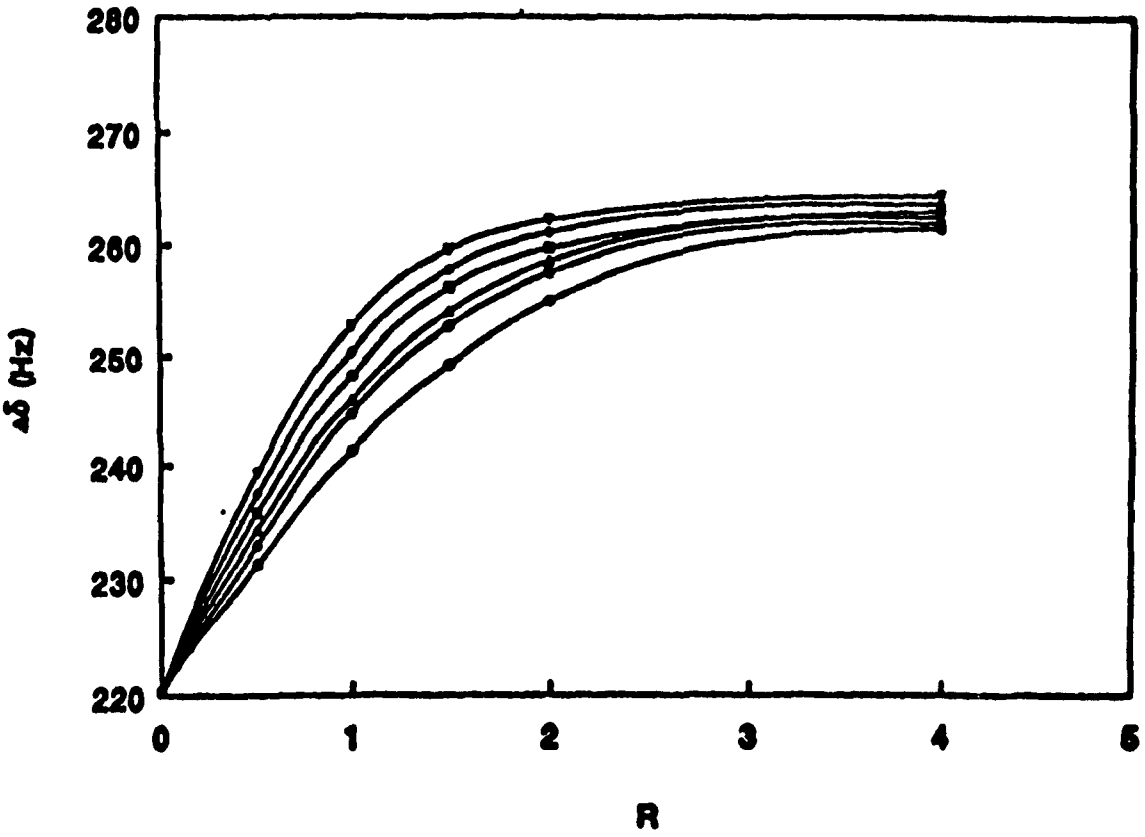


Figure 5.14. The plots of the chemical changes for 3' protons of ß-cyclodextrin in the presence of: (▲) DNP-L-valine, (●) DNP-D-valine, (▼) DNP-L-leucine, (◆) DNP-D-leucine, (■) DNP-L-methionine, and (●) DNP-D-methionine. R is the molar ratio of DNP-amino acid to ß-cyclodextrin.

5.3.3.2. Effect of ß-cyclodextrin on ^{1}H NMR spectra of DNP-DL-amino acids.

The effect of ß-cyclodextrin on the ^{1}H NMR spectra of DNP-amino acids was studied by setting the concentration of DNP-amino acid at 0.005 M and varying the molar ratio of ß-cyclodextrin to DNP-amino acid from 0 to 3.0. It is found that in the presence of ß-cyclodextrin, the resonance of the aromatic 6H is shifted to lower field, and no significant difference can be observed between D- and L-enantiomers. Downfield proton resonance shifts of a molecule can usually be observed when this molecule binds to another by interactions of dipole-dipole, dipole-induced dipole, and London, which physical chemists have referred to as van der Waals forces [47], by steric perturbation [48], or by diamagnetic anisotropy of particular bonds or regions of the host molecule [49]. In this case, the down field of aromatic 6H may be mainly caused by the van der Waals interactions with the hydrophobic cavity of ß-cyclodextrin.

In contrast, for all the DNP-amino acids, the chemical shift of aromatic 3H, which is located between the two nitro substituents, is moved upfield with inclusion complexation with ß-cyclodextrin.

No significant chemical shift change was observed for the aromatic 5H. This fact further confirms the inclusion complex structure predicted by the CD studies. As shown in Figure 5.10, the DNP group of the guest molecules is included into the ß-cyclodextrin cavity in such a way that the 6H is closer to the wall of the cavity than the 5H. It is known that van der Waals forces are extremely short-range, being proportional to $1/r^6$ [50], where r is the distance between the two interaction groups. Therefore, the interactions between the cavity wall and 5H are very weak, and so no substantial chemical shift change

is expected for this proton upon the inclusion complex formation with ß-cyclodextrin.

In the presence of ß-cyclodextrin, substantial chemical shift changes were also observed for alkyl protons of both D- and L- DNP-amino acids. However, the shift changes are more significant for the alkyl protons of the L-enantiomers. For example, the chemical shifts of the methyl protons moved 6.62 Hz to lower field for both DNP-L-leucine and DNP-L-valine after inclusion complex formation with ß-cyclodextrin (R = 1), while the changes are only 1.1 Hz and 1.64 Hz for DNP-D-leucine and DNP-D-valine, respectively. This kind of behaviour may be indicative of a secondary inclusion complex formation between the smaller side of ß-cyclodextrin cavity and the methyl group of the DNP-L-amino acids. The linewidth for the resonances of alkyl protons also increased significantly with increasing ß-cyclodextrin concentration. Although perfectly quantitative line width comparison is impossible due to the lack of an internal line width standard, it is clear that the resonance lines assigned to the protons in the vicinity of the chiral center are broadened more significantly then those of the protons far from the chiral center. The shift changes and line broadening may be caused mainly by the steric perturbations upon inclusion complex formation with ß-cyclodextrin.

It should be pointed that the host-guest system is in the NMR chemical-shift fast-exchange limit [51, 52]. In this case, the measured resonance positions for the protons of DNP-amino acid appear as the average of the chemical shift of free DNP-amino acid and the chemical shift of DNP-amino acid bound in each possible orientation to ß-cyclodextrin, weighted by the fractional population of DNP-amino acid molecules in each environment. For ß-cyclodextrin protons, the measured resonance positions are the average of the chemical shift of 'empty' ß-cyclodextrin molecules and the chemical shift of ß-cyclodextrin molecules which have guests, weighted by the fractional

population in each state.

5.3.4. The dissociation constants of ß-cyclodextrin-DNP amino acid inclusion complexes.

All the dissociation constants determined by UV-visible, CD, and NMR methods are listed in Table 5.1, along with the retention data obtained on the ß-cyclodextrin bonded phase column.

The K_d values listed in Table 5.1 show large deviations between different determination methods. As can be seen, the K_d values measured by NMR spectroscopy are about as twice large as that determined by UV method. This difference is mainly attributed to the different media used for the measurements. UV measurements, as mentioned in the experimental section, were carried out in a pH 6.0 phosphate buffer while NMR measurements were done in a pH 11.0 phosphate buffer. The results demonstrated that the stability of the ß-cyclodextrin-DNP-amino acid inclusion complexes decreased with increasing pH. The K_d values obtained by CD method, which mostly reflect interactions extent between the carboxyl group of amino acid and the ß-cyclodextrin, also show significant differences from the UV results.

Although large K_d deviations exist between the determination methods, it is clear that ß-cyclodextrin did form enantioselective inclusion complexes with DNP-amino acids. The K_d values determined by all of these three method clearly indicate that the inclusion complex formed between ß-cyclodextrin and DNP-L-amino acid is more stable than the corresponding ß-cyclodextrin-DNP-D-amino acid inclusion complex. This fact coincides with the chromatographic data which show that the DNP-L-amino acid always has longer retention times than its D-enantiomer on a ß-cyclodextrin bonded phase column.

5.3.5. Structure of the inclusion complexes and chiral recognition mechanism.

In principle, DNP-amino acids can penetrate the ß-cyclodextrin cavity in only two orientations, either the amino acid part first or the *para*-nitro group of the DNP substituent first. The orientation of inserting the *ortho*- or *meta*- position of the DNP substituent is impossible because very little of the substituent would actually fit onto the cavity. The CD and ^{1}H NMR studies clearly suggest that the penetrations for both D- and L-DNP-amino acids are by the para-nitro group head-on into the cavity from the wide 2, 3-hydroxyl side, with the amino acid part sticking out, as shown in Figure 9. It is observed that the depth and the tilt of the aromatic ring in the cavity are significantly different for the D- and L-enantiomers. The lower intensity of the induced bands for DNP-L-amino acids indicate that DNP-L-amino acids have a large tilt angle in the cavity than their D-enantiomers. The large angle may be caused by the formation of strong hydrogen-bonding between the carboxyl group of DNP-L-amino acid and the hydroxyl group on the edge of ß-cyclodextrin cavity. The larger chemical shift change observed for the 5' protons of ß-cyclodextrin in the presence of DNP-D-amino acid suggests that the insertion is deeper for DNP-D-amino acid than for DNP-L-amino acid because the large tilt of DNP group in the latter case hinder it from penetrating further. The shallower insertion for DNP-L-amino acids leave enough space in the ß-cyclodextrin cavity to host the alkyl group from another DNP-L-amino acid. In the presence of ß-cyclodextrin, the large chemical shift changes observed for the alkyl protons of DNP-L-amino acids are direct evidence for the existence of secondary inclusion complex formation between the ß-cyclodextrin and the alkyl groups.

Based on observations and the above discussions, the proposed structure

of ß-cyclodextrin complex with DNP-L-amino acids is in Figure 5.15-A, where two or even more inclusion complexes of 1:1 stoichiometry are associated. In the primary 1:1 complex, the dinitrophenyl portion of DNP-L-amino acid is included within the cavity and the carboxylic group interacts with the hydroxyl group at the edge of the cavity through hydrogen bonding, leaving the alkyl group to insert into another cyclodextrin cavity from its smaller 6-hydroxyl side.

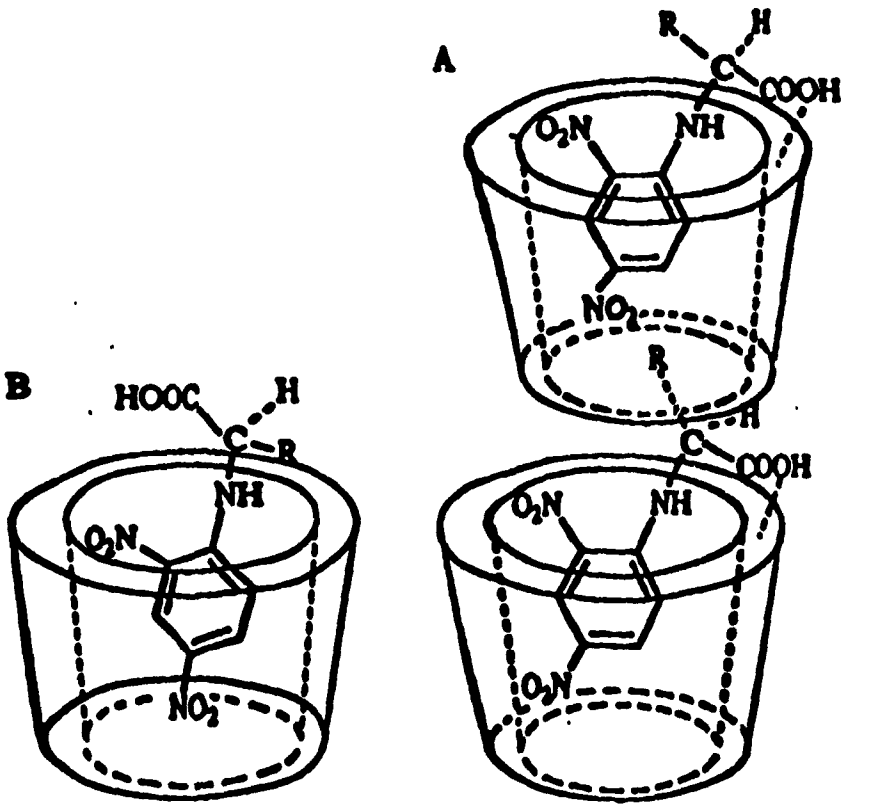


Figure 1.15. Structures of inclusion complexes of ß-cyclodextrin with DNP-D-amino acid, and with DNP-L-amino acid.

Figure 5.15-B is the proposed structure of ß-cyclodextrin with DNP-D-amino acids. In this case, the repulsion between alkyl group of DNP-D-amino acid and the hydroxyl groups at the edge of ß-cyclodextrin cavity results in a smaller tilt angle and deeper penetration of the DNP group in the cavity, and leaves no space to hold the alkyl group in the cavity. On the other hand, since the alkyl groups of DNP-D-amino acids are held tightly against the edge of ß-cyclodextrin cavity, even if there were enough space in the cavity, it is difficult to form a secondary inclusion complex due to the steric hindrance. Therefore, no secondary inclusion complex can be formed between ß-cyclodextrin and the alkyl groups of DNP-D-amino acids.

These proposed structures for DNP-L-amino acids and DNP-D-amino acids show the precise nature of the chiral discrimination interaction and chirality forces responsible for the chiral resolutions. It appears that the DNP group, which forms a stable inclusion complex with ß-cyclodextrin cavity and places the other functional groups around the chiral centre in association with the polar hydroxyl groups at the edge of the cavity, plays a very important role in the chiral recognition. The alkyl groups of amino acids, which could form secondary inclusion complex with another ß-cyclodextrin cavity (in the case of DNP-L-amino acids) or play a role of steric repulsion with the hydroxyl groups at the edge of the cavity (in the case of DNP-D-amino acids), are also major contributors to the chiral recognition.

5.4. CONCLUSION

UV, CD, and 1H NMR studies give detailed information about the stability and structures of the inclusion complexes of ß-cyclodextrin with DNP-L-amino acids and DNP-D-amino acids. Such direct spectroscopic observation of the 'soluble models of the chromatographic system' substantiate the

presumed chiral recognition mechanism and add further details as to conformational preference during inclusion complex formation. The results demonstrate that the formation of an inclusion complex is an essential requirement for the chiral separation, but it is also clear that only inclusion complexation is not sufficient for chiral recognition. Sufficient chiral recognition also requires the interactions of other functional groups around the chiral centre with the mouth of ß-cyclodextrin cavity and/or with another cyclodextrin cavity to form a secondary inclusion complex.

Compared with the theoretical studies [53] and computer modelling methods [54, 55], these spectroscopic studies account better for the contribution of mobile-phase composition, but still neglect the intricate effect of the silica surface. Together with the chromatographic data in a previous report [41], these spectroscopic studies give a clearer picture for the chiral recognition process, and will aid the design of new chiral stationary phases to meet specific chiral separation problems.

5.5. REFERENCES

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CHAPTER 6

A MULTIPLE-INTERACTION CHIRAL STATIONARY PHASE BASED ON THE MODIFIED ß-CYCLODEXTRIN BONDED STATIONARY PHASE

6.1 INTRODUCTION

As was presented in Chapter 2, direct separation of enantiomers with HPLC techniques has been a very active and fast-moving field in recent years due to its importance in the optical purity determination, monitoring asymmetric synthesis, pharmacokinetics studies, metabolism studies, and dating archaeological materials. The use of HPLC with chiral stationary phases has become the most common first approach for enantiomer separation. Today, more than 50 different chiral stationary phases are commercially available. These stationary phases can be classified into five categories [1, 2]: donor-acceptor phases, chiral cavity phases, ligand-exchange phases, helical polymer phases and protein phases.

Among these stationary phases, donor-acceptor chiral stationary phases, for which the chiral recognition involves hydrogen-bonding, π-π (or charge-transfer) interaction and steric hindrance, have perhaps a wider demonstrated applicability than any other class of chiral stationary phases. However, they

have also some limitations and/or restrictions [1]. The chief limitations are as follows:

(i). The frequent and, in some respects, inherent need for derivatizations. Chiral resolution requires the solute to have at least one π-acidic or π-basic aromatic group.

(ii). The requirement for relatively lipophilic solutes. Strongly anionic molecules, such as carboxylic acids, and strongly cationic molecules, such as amines, are not easily resolved on these chiral stationary phases.

(iii). The requirement that at least one of the interacting functional groups be proximate to the chiral center.

Cyclodextrin bonded stationary phases, especially ß-cyclodextrin stationary phase, developed by Armstrong [3], is another type of widely used chiral stationary phases. Compared to other chiral stationary phases, ß-cyclodextrin stationary phase has the following advantages:

(i) it is chemically and physically much more robust;

(ii) it can be operated in a reversed-phase mode with solvents containing water and organic modifiers; and

(iii) it is cheaper than any other chiral stationary phase.

However, ß-cyclodextrin stationary phase has a limited range of applications. Chiral recognition is significant only for those molecules which have a large hydrophobic group in the molecule. This limitation is mainly related to the nature of ß-cyclodextrin itself and the chiral recognition mechanism.

As was described in Chapter 1, the structure of ß-cyclodextrin has a shape of a toroid or hollow truncated cone. The side of the torus with the larger circumference contains the secondary hydroxyl groups (on carbons 2 and

3 of the glucose units) while the primary hydroxyl groups (on carbon 6 of the glucose units) are on the small side. The interior of the cavity contains two rings of C-H groups with a ring of glycosidic oxygens in between. As a result, the cavity is relatively hydrophobic while the external faces are hydrophilic [4]. In reverse-phase applications, chiral recognition is believed to be mainly caused by hydrophobic interaction between the cavity of cyclodextrin and the hydrophobic moiety of the solute. The chief problem is that because the interior of the cavity, with glycosidic oxygens, is not quite nonpolar and both ends of the cavity are open to solvent, only those guest molecules which have large hydrophobic groups and appropriate shape can form strong inclusion complexes with it. Therefore, chiral recognition is significant only for these larger molecules.

To overcome this problem, much work has been done to increase the binding forces by modifying the cyclodextrins through the reaction of the hydroxy groups with variety modifiers [5, 6]. But, to date, no significant improvement in stereoselectivity has been achieved. The reason may be that the non-specific modification (both primary and secondary hydroxyl groups were derivatized) blocks both sides of the cyclodextrin or decreases the hydrophillic properties of the outside surface of the cyclodextrin, resulting in a decrease in the ability of inclusion complex formation.

In this work, a regiospecific modification was carried out by attaching groups on the primary hydroxyl side of the ß-cyclodextrin. This modified ß-cyclodextrin stationary phase contains:

(i) an hydrophobic cavity, capable of inclusion complexation;
(ii) aromatic groups, capable of π-π interaction;
(iii) polar hydrogen-bonding and/or dipole-stacking sites; and
(iv) bulky non-polar groups, providing steric repulsion, van der Waals

interaction, and/or confirmational control.

Therefore, it can be said that it is a multiple-interaction type of chiral stationary phase.

The enantiomeric separations of various compounds including amino acid derivatives, phenothiazine and related drugs, and other pharmaceuticals have been achieved on these multiple-interaction chiral stationary phases. Some separations achieved on these phases were impossible on the conventional ß-cyclodextrin stationary phases.

6.2. EXPERIMENTAL

6.2.1. Materials

6.2.1.1. Chemicals

ß-Cyclodextrin was from Chemical Dynamics Co. (South Plainfield, New Jersey). HPLC grade methanol and triethylamine were from Fisher Scientific (Montreal, Quebec). N-(2-aminoethyl-3-aminopropyl)-trimethoxysilane was from Huls America Inc. (Bristol, Pennsylvania). Amino acids and their derivatives, and the phenthiazines were from Sigma (St. Louis, MO). All other chemicals were from Aldrich (Milwaukee, Wisconsin).

6.2.1.2. The Silica Gel

A silica gel purchased from Chromatographic Separation Company (Montreal, Canada) as SPHERISORB was used as the support material. This gel consists of spherical particles with a pore diameter of 8 nm and a mean particle size of five microns. The surface area of the silica gel, according to the manufacturer, is 220 m^2/g. Before carrying out reactions, the silica support was

acid-hydrolysed in 0.2 M HCl at 90°C for 24 hr, so that its surface has a maximum number of Si-OH groups per unit surface area. After cooling and filtration, the silica was washed with distilled water until neutral, and then dried at 170°C *in vacuo* for 12 hr.

6.2.1.3. Apparatus

All chromatographic experiments were performed on a liquid chromatographic system which consisted of a Model 590 pump (Waters Assoc., Milford, MA), a Model 440 254-nm ultraviolet detector (Water Assoc.) and a Model 7125 injector containing a 10-μL loop (Rheodyne, Cotati, CA). Injection were made on column using precision sampling syringes. All column evaluations were carried out at ambient temperature (ca 20°C).

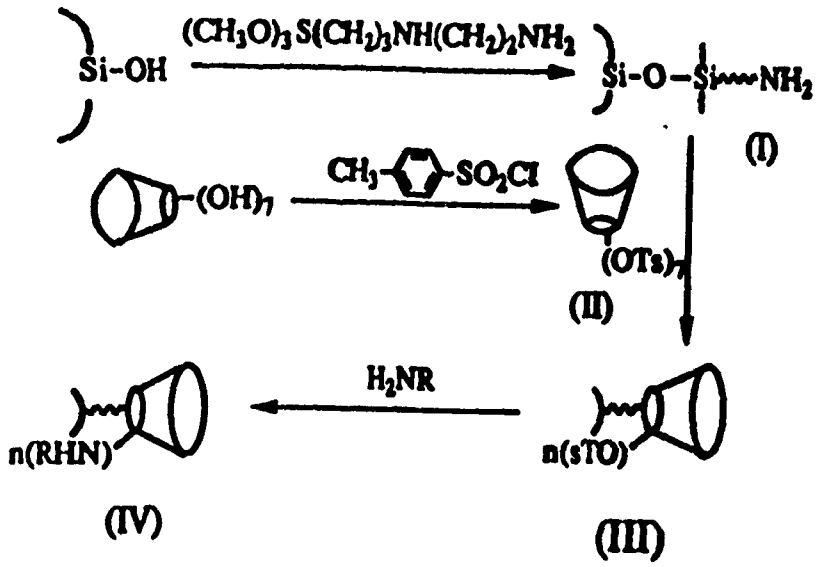
The column were 25-cm lengths of 0.655 cm o.d. x 0.46 cm i.d. stainless-steel tubing (Chromatographic Separation Co., Montreal, Quebec) with a mirror-finish. All stainless-steel fittings were modified for minimum dead volume and clean flow-through patterns. Column blanks and all tubing were carefully cleaned before use.

6.2.2. Preparation of the multiple-interaction stationary phases

6.2.2.1. General approaches

The preparation procedures used in this investigation are shown in Figure 6.1. Two approaches have been tried for the preparation of packing materials. In the first approch, the ß-cyclodextrin was bonded to the silica gel through a procedure described by Fujimura and coworkers [7]. In the second approch, the ß-cyclodextrin was bonded to the silica gel through Armstrong's procedure [3]. Comparing these two approaches, the first one is much more easily carried out. Here, only the first approach is described. It involves four steps:

Procedure 1:



Procedure 2:

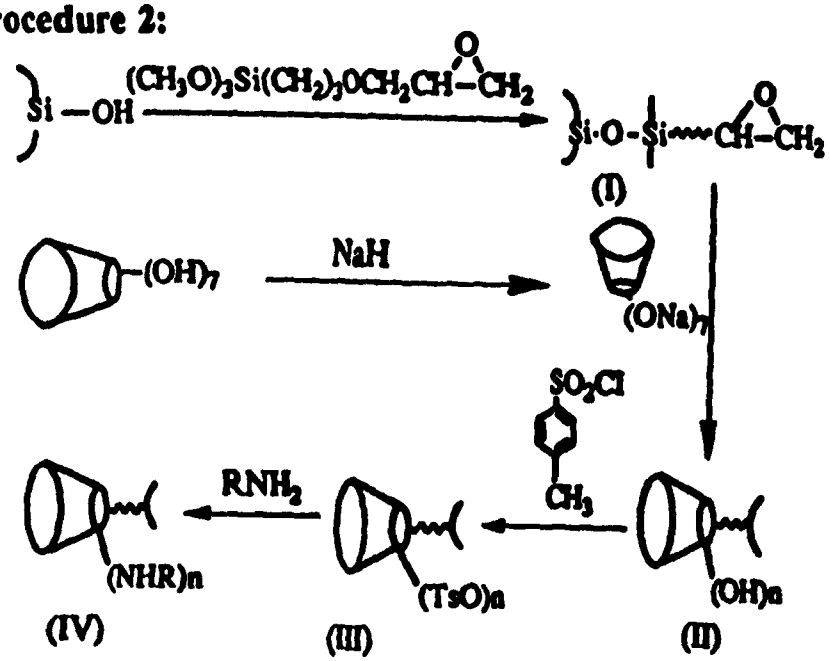


Figure 6.1. General procedures for the preparation of the modified β-cyclodextrin stationary phases.

(i). Bond the N-(2-aminoethyl-3-aminopropyl)trimethoxysilane to the silica gel. N-(2-aminoethyl-3-aminopropyl)trimethoxy silane was used as a linkage material to join the ß-cyclodextrin to the silica.

(ii). Regiospecific sulfonation of the primary hydroxyl groups of the ß-cyclodextrin with *p*-toluenesulfonyl chloride.

(iii). Reaction of 2-aminoethyl-3-aminopropyl bonded silica gel (I) with toluenesulfonyl-ß-cyclodextrin (II). The reaction between the amino groups of the N-(2-aminoethyl-3-aminopropyl) bonded silica and the toluenesulfonyl groups of the cyclodextrin will link the cyclodextrin to the silica gel.

(iv). Modification of the bonded cyclodextrin by further reaction of the unreacted toluene sulfonyl groups of the cyclodextrin (III) with various modifiers.

6.2.2.2. Reaction of N-(2-aminoethyl-3-aminopropyl)trimethoxysiliane with silica gel

The following procedure is a typical reaction used to modify the surface of various porous silica particles with hydrocarbon groups:

Ten grams of acid-hydrolized 5-µm SPHERISORB silica gel, which had been dried *in vacuo* at 170°C overnight, was paced in a 500 ml three-neck flask with a magnetic stirrer. To this flask were added 200 ml of dry toluene treated with Type 4A molecular sieve and 11.1 grams (0.05 mole) of N-(2-aminoethyl-3-aminopropyl)trimethoxysilane. A reflux condenser fitted with a calcium chloride drying tube was attached to the flask and a slow stream of dry nitrogen was passed through the flask via a capillary tube inserted in one of the side necks. This mixture was refluxed while stirring slowly for 10 hours, after which time the reaction mixture was cooled to room temperature. The

solids were isolated with a fritted glass filter, washed thoroughly with dry toluene to remove any residual N-(2-aminoethyl-3-propyl)trimethoxysilane then washed with 40 ml of methanol. The isolated functionalized silica gel was then dried *in vacuo* over P_2O_5 at 60°C.

6.2.2.3. Regiospecific sulfonation of the primary hydroxyl groups of the ß-cyclodextrin [8]

Five grams of ß-cyclodextrin (dried *in vacuo* at 100°C over P_2O_5 for 12 hours) was dissolved in 60 ml of anhydrous pyridine. To this mixture was added with stirring 5.85 grams of toluenesulfonyl chloride (the molar ratio of ß-CD to TsCl is 1:7), and the mixture was let stir at room temperature. After 24 hours, 2 ml of water was added to the mixture, and solvent was removed under vacuum until a viscous residue remained. The residue is dissolved in 150 ml of 65°C water, and let stand at 4°C overnight. The solid product is collected and further purified by recrystallizing several times in methanol. The product is then dried *in vacuo* at 60°C.

Identification:

Molecular weight $(C_{13}H_{16}O_7S)_7$, 2214.27; [α] 20/D in chloroform (1%), +105 ± 1; [α] 20/D in methanol (1%), +96° ± 1; NMR (in CD_3Cl) gives: 7.74 (*meta*-H of toluene group), 7.29 (*ortho*-H of the tolunene group), 4.72 (1' H of ß-cyclodextrin), 4.45 (6' H of ß-cyclodextrin), 4.14 (3' H of ß-cyclodextrin), 3.74 (5' H of ß-cyclodextrin), 3.58 (2' H of ß-cyclodextrin), 3.32 (4' H of ß-cyclodextrin), and 2.37 (for the protons of toluene methyl group).

6.2.2.4. Reaction of amine-type silica gel with toluenesulfonyl-ß-cyclodextrin

Three grams of dried 2-aminethyl-3-aminopropyl bonded silica gel was placed in a 250 ml three-neck flask. To this flask were added a magnetic stirring bar, 100 ml of dry pyridine (or dimethylformamide) and four grams of toluenesulfonyl-ß-cyclodextrin. The mixture was stirred at 90°C for 5 hours. After 5 hours, 0.05 mole of modifying agents was added. The mixture was stirred at 90°C. After 10 hours, the reaction mixture was cooled to room temperature, filtered with a fritted glass funnel and washed several times with DMF and methanol. The isolated functionalized silica was air-dried.

6.2.3. Analyses of the surface species

Identification of the surface species is made by means of infrared spectroscopy using transmission and the attenuated total reflection techniques. In this investigation, the amounts of cyclodextrin bonded to the silica gel were determined by means of a colorimetric method based on the reaction of D-glucose with tetrazolium blue [7, 9]. The stationary phase (0.5 g) was hydrolysed in 30 ml of 0.5 M sulfuric acid at 100°C for 5 hours. The hydrolysate was neutralized with sodium hydroxide and made up to 50 ml with vater. In a reaction vessel with a glass stopper, 1 ml of this solution was reacted at 50°C for 10 min with 1 ml of 0.5% tetrazolium blue 4:1 ethanol-water solution of 0.2 M NaOH. After the vessel was cooled, 3 ml methanol was added, and Na_2SO_4 was removed by centrifugation. The absorbance of the clear solution was measured at 525 nm.

6.2.4. Column Packing Technique

Columns were prepared by a high-pressure balanced-density slurry packing technique using a Shandon HPLC packing pump (Chromatographic Separation Co., Montreal, Quebec) with a 33 ml slurry reservoir. The diagram of this packing pump is shown in Figure 6.2. Packing slurries were prepared by placing 3 grams of the dried packing in 30 ml of 2-propanol and shaking ultrasonically until a homogeneous slurry is produced. The slurry was then poured into the packing reservoir. The column blank prepared from stainless steel and a reducing union with a 2-μm frit was attached to the slurry reservoir. The packing process involved the following steps:

(i) pump upstream 100 ml of n-hexane at a packing pressure of 9000 psi;

(ii) invert the column-packing reservoir combination, allow the system to stand until a further 40 ml of n-hexane was pumped;

(iii) change the solvent to methanol, pump 40 ml of methanol.

All packed columns were purged thoroughly with methanol then equilibrated with the appropriate mobile phase before use. When not in use, the columns were stored in methanol.

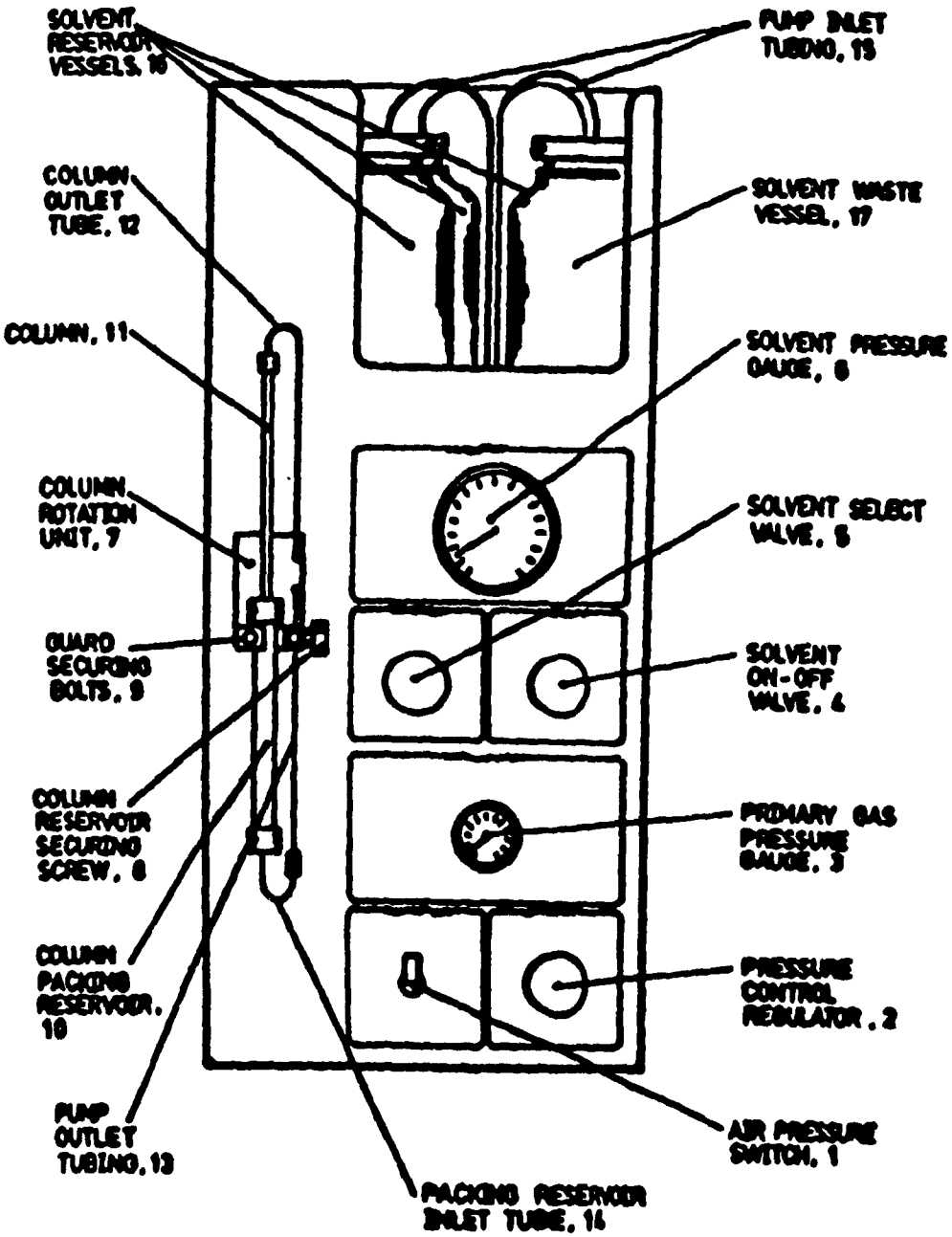


Figure 6.2. Diagram of Shandon HPLC packing pump.

6.3. RESULTS AND DISCUSSION

6.3.1. Preparations of the packings

Two procedures have been tried for the preparation of the multiple-interaction chiral stationary phases in this investigation. In the first procedure, as was described in the experimental section, a linkage material, N-(2-aminoethyl-3-aminopropyl) trimethoxysilane, was first bonded to the silica gel. The cyclodextrin was joined to the silica through the reaction of this amine-modified silica gel with the cyclodextrin tosylate. The linkage occurs only through the primary hydroxyl groups at the small side of cyclodextrin. The final multiple-interaction stationary phases were produced by further reaction of the unreacted toluenesulfonyl groups on the cyclodextrin. To date, four different modified ß-CD stationary phases have been developed (see Figure 6.3). Although one linkage is shown between cyclodextrin and the silica, the formation of two or more bonds is possible.

In the second procedure, the coupling of cyclodextrin to silica gel is accomplished by the reaction between 3-glycidoxypropyl trimethoxysilane silanized silica gel and a sodium hydride treated cyclodextrin. This cyclodextrin bonded stationary phase was then tosylated by reacting with p-toluenesulfonyl chloride. The final packing materials were produced by the reaction of various modifiers with this tosylated cyclodextrin bonded silica.

While both procedures produce fairly stable bonds, the first one seems much easier to carry out. The reproducibility of the second procedure requires rigorous control of reaction conditions.

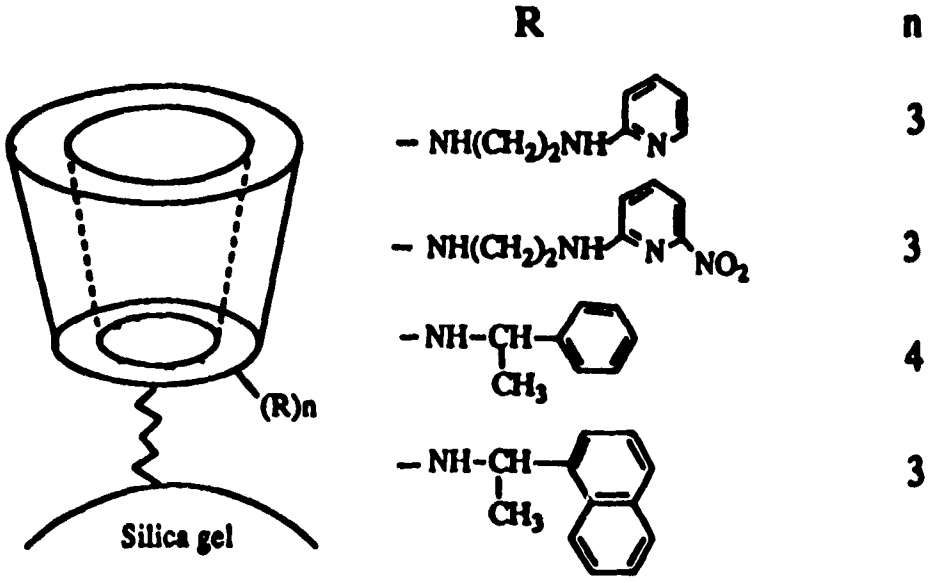


Figure 6.3. The structures of modified ß-cyclodextrin stationary phases.

6.3.2. Characteristics of the packings

6.3.2.1. Surface coverage

The most valuable parameter for characterization of bonded-phase packings is the surface concentration of bonded functional groups. However, most of the commercial chiral stationary phases are only characterized by their carbon content, which gives poor and incomplete information. In general, it is very difficult to precisely and/or accurately evaluate the content of the cyclodextrins bonded to the surface of silica gel by the method based on differences in the gel weights before and after the bonding reaction or by elements analysis. In this work, the amount of cyclodextrin bonded to the silica gel was colorimetrically determined by a method based on the reaction of D-

glucose produced by hydrolysing cyclodextrins with 3,3'-[3,3'-dimethoxy-(1,1'-biphenyl-4,4'-diyl)]bis(2,5-diphenyl-2H-tetrazolium)dichloride, called tetrazolium blue. The maximum coverage for these stationary phases was determined to be 50.6 µmole/g.

To determine the degree of the substitution on the ß-cyclodextrin of the modified ß-cyclodextrin stationary phases, an HPLC method has been used. The degree of substitution for each of the stationary phases was calculated and is reported in Figure 6.3.

6.3.2.2. Stability

The chemical stability of the stationary phase is primarily determined by that of silica and the bonded cyclodextrins. Consequently, most solvents can be used if they are within the pH range of 3.0 -7.0. Although cyclodextrin molecules and their derivatives are fairly stable in alkaline solution, they are quite susceptible to hydrolysis in strongly acid solution. For example, the hydrolysis rate constants of hydrolysis of ß-cyclodextrin at pH = 0.133, 40°C and 100°C, are 1.0×10^{-6} and 4.8×10^{-2} min^{-1}, respectively, corresponding to half-lives of 48 days and 14 min [4]. The stability in aqueous solutions of pH > 7 is influenced by the presence of organohydroxylsilyl groups resulting from the hydrolysis of $Si\text{-}(OCH_3)_3$ or $Si\text{-}(OC_2H_5)_3$ groups of the linkage materials. These stationary phases can be used at temperature up to 65°C.

6.3.3. Chromatographic Properties

6.3.3.1. Column Efficiency

Each of the slurry-packed modified ß-cyclodextrin columns was conditioned with a solvent series of increasing polarity. This series consisted of isopropanol, methanol and 50:50 methanol/water. After conditioning, all columns were evaluated using a test mixture of o-chlorophenol, p-chlorophenol, 3-nitroaniline, and 2-biphenylol and a mobile phase of 50:50 methanol/water. The HETP values obtained on two of these columns at flow-rates of 0.5 ml/min and 1.0 ml/min are listed in Table 6.1. As can be seen, The efficiencies of these modified columns are lower than that of the unmodified ß-cyclodextrin column.

Like the conventional ß-cyclodextrin columns, the efficiency of these modified ß-cyclodextrin columns is dramatically improved by adding TEAA (triethylammunium acetate) buffer in the mobile phase. As shown in Figure 6.4, the presence of 0.3% TEAA in the mobile phase can produce a three-fold increase in the column efficiency.

Table 6.1.
Column efficiency (HETP values)

COLUMNS	0.5 ml/min	1.0 ml/min
β-CD	0.0014 cm	0.0015 cm
β-CD - NH-CH(CH$_3$)-	0.0035 cm	0.0040 cm
β-CD - NH-CH(CH$_3$)- 	0.0039 cm	0.0046 cm

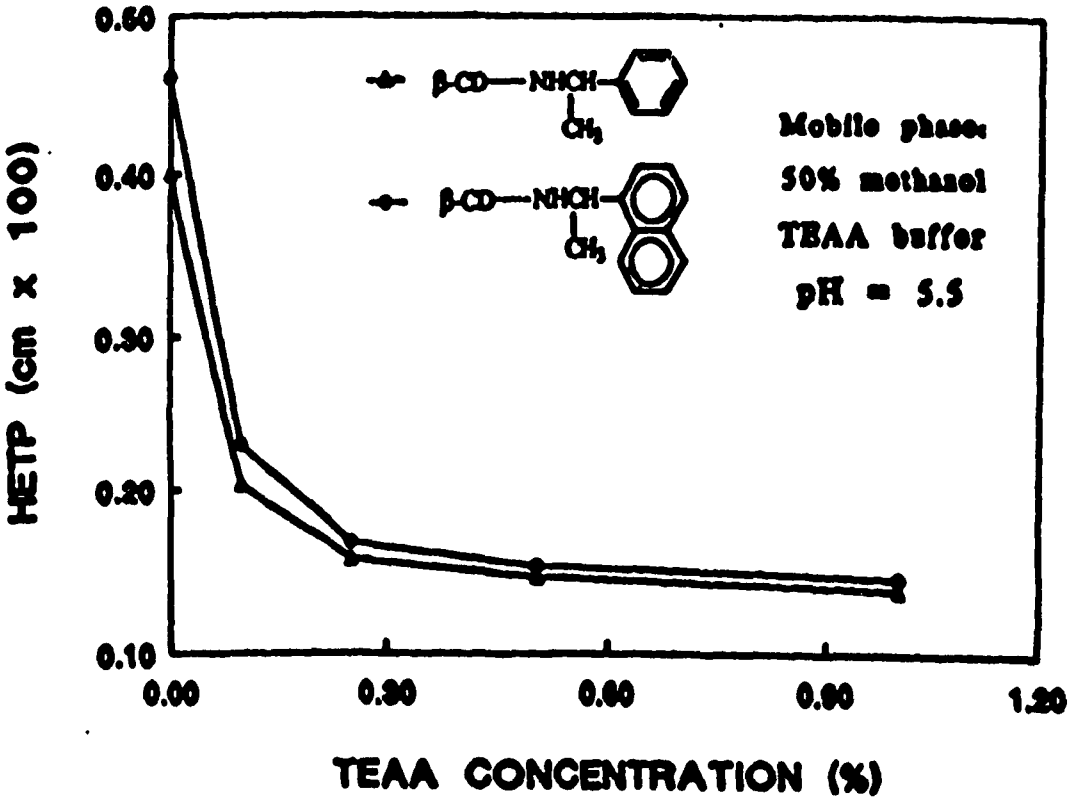


Figure 6.4. Effect of TEAA on the column efficiency.

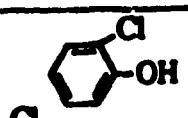
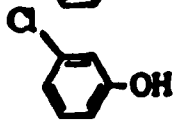
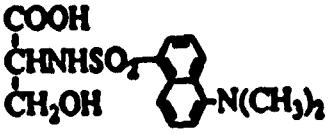
6.3.3.2. Retention time

For most solutes, the retention time on these multiple-interaction stationary phases is much longer than the retention time on the ß-cyclodextrin bonded stationary phase. For instance, the capacity factors of o-, m- and p-chlorophenols are more than two times larger than those on the ß-cyclodextrin stationary phase (see Table 6.2). The longer retention time means that the modification indeed increases the binding forces of inclusion complex formation

between the guest and the cyclodextrin.

However, the retention time for dansyl- amino acids is shorter than that obtained on the ß-cyclodextrin column. The reason for this behaviour is that the modification may make the cyclodextrin cavity too shallow, so that the large naphthylamine group cannot completely fit into the cavity. The strength of the inclusion complex is, therefore, decreased.

Table 6.2.
Capacity factors on ß-cyclodextrin and modified ß-cyclodextrin columns

Solutes	Capacity Factors		
	β-CD	β-CD–NHCHCH$_3$	β-CD–NHCHCH$_3$
	0.87	2.56	2.32
	1.00	2.35	2.15
	1.10	2.16	2.01
	2.06	1.71	1.83

6.3.3.3. Enantioselectivities

It has been found that these multiple-interaction stationary phases exhibited higher enantioselectivity toward a wider variety of chiral compounds. The improved enantioselectivity may be rationalized in terms of the following two factors.

(1). The attached groups on the primary hydroxyl side of ß-cyclodextrin cavity increase the capability of inclusion complexation with a wide variety of compounds. As mentioned in Chapter 1 and reported in Chapter 5, to achieve chiral separation on a ß-cyclodextrin stationary phase, the guest molecule must form a enantioselective complex with ß-cyclodextrin. Typically, cyclodextrin inclusion complex formation is associated with a favourable enthalpy and an unfavourable entropy change [4, 10]. Although the exact nature of the bonding forces involved in the complex formation still remains controversial, several proposals have been made to interpret the binding forces between the guest and the cyclodextrin molecule in solution [10-13]:

(i) van der Waals-Landon dispersion forces (the so-called "hydrophobic effect");
(ii) hydrogen bonding between the polar group of the guest molecule and the secondary hydroxyl groups of the cyclodextrin;
(iii) release of high energy water molecules in complex formation; and
(iv) release of the strain energy in the ring frame system of the cyclodextrin.

In most cases, a combination of these factors seems to be operative, with the first dominant. However, because the interior of the cavity, with a ring of ether oxygens, is not quite non-polar and both ends of the cavity are open to solvent,

only those guest molecules which have large hydrophobic groups and appropriate shape can form strong inclusion complexes with it.

For these modified ß-cyclodextrin stationary phases, the attached groups on the primary hydroxyl side can cluster to form a hydrophobic "floor" on the ß-cyclodextrin cavity [14] (see Figure 6.5). This will increase the hydrophobicity of the cavity, thus increasing the capability of the inclusion complex formation with the smaller molecules. It was found that the solutes which have only one benzyl ring in the molecule were indeed bound strongly on these modified ß-cyclodextrin stationary phases as shown by the increased retention time on these columns.

(2). These stationary phase provide multiple-interaction sites which increase the number of specific, discrete and simultaneous interactions between chiral solute molecules and the stationary phase. This modified ß-cyclodextrin stationary phase has the following functional groups:

(i) an hydrophobic cavity, capable of inclusion complexation;
(ii) aromatic groups, capable of π-π interaction;
(iii) polar hydrogen-bonding sites, capable of hydrogen-binding with the polar functional groups on the chiral solutes; and
(iv) bulky non-polar groups, providing steric repulsion.

It has been believed that the greater the number of specific, discrete, simultaneous interactions between chiral solutes molecules and a chiral locus on the stationary phase, then the greater the likelihood of effective chiral discrimination, and thus of chromatographic resolution of enantiomeric solutes [15].

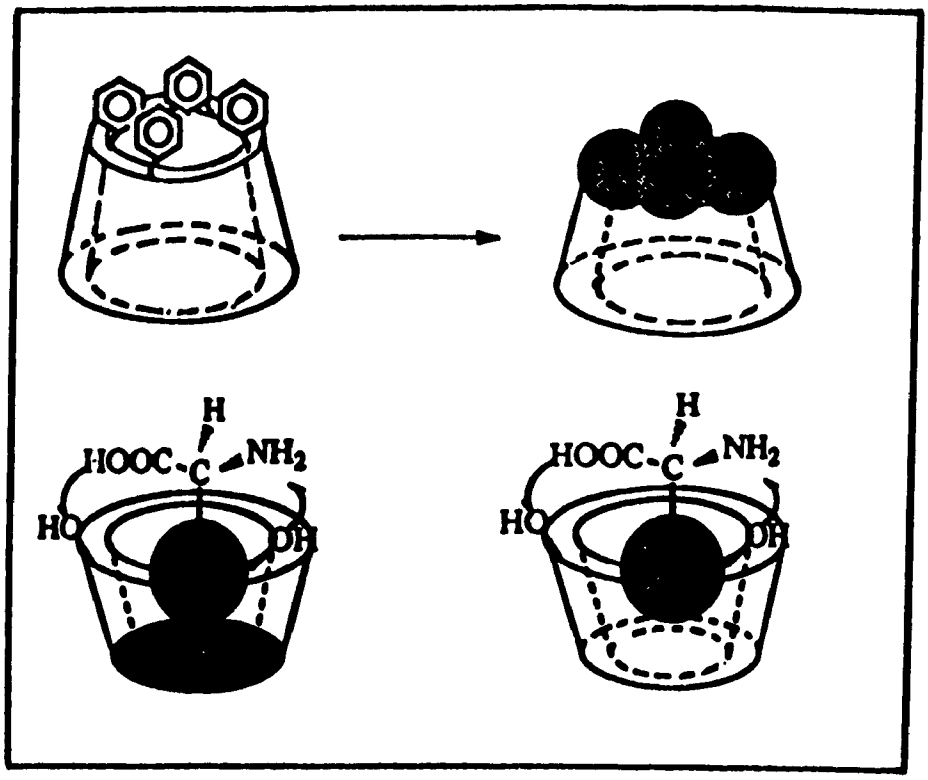


Figure 6.5. Structural diagram of modified ß-cyclodextrin. The attached aromatic groups on the primary hydroxyl side cluster to form a hydrophobic "floor" on the cyclodextrin cavity. The solutes which have only one benzyl ring in the molecule are bound strongly on it.

6.3.4. Enantiomeric separations

These modified stationary phase have demonstrated a broader range of applications. The enantiomeric separations of various compounds including amino acids and their derivatives, carboxylic acids, phenothiazine and related drugs, and other pharmaceuticals have been achieved on these modified ß-CD columns.

6.3.4.1. Enantiomeric separation of dansyl-amino acids

1-Dimethylaminonaphthalene-5-sulfonyl amino acids (dansyl amino acids) occupy a key position in the structural investigation of proteins and peptides, and the quantitative analysis of amino acids. Dansyl chloride reacts with free amino acids and phenolic groups and is increasingly used in determining the amino-terminal residues of protein and peptides [16].

In this work, the enantiomeric separation of 10 dansyl amino acids have been achieved on the methylbenzylamine modified ß-cyclodextrin stationary phases using methanol-TEAA buffer solution (0.3% TEAA) as the mobile phase. Table 6.3 shows the optical resolution data for these 10 dansyl amino acids. All 10 chiral compounds can be optically separated with resolution factors from 0.74 to 1.30. However, for most dansyl amino acids, the resolution factor is lower than that achieved on the unmodified ß-cyclodextrin column. The reason, as mentioned before, is that dansyl amino acids cannot form strong inclusion complexes with modified ß-cyclodextrin stationary phases.

Figure 6.6 shows some typical chromatograms for the solutions of these racemic dansyl amino acids. The L-enantiomers are eluted first for all the dansyl amino acids. The enatiomeric separations of dansyl amino acids seems to have considerable potential as an analytical method for the steric structural investigation of protein and peptides.

Table 6.3.
Optical Resolution of the Enantiomers of Dansylamino Acids

	β-CD—NH—CH(CH$_3$)—				β-CD
Solutes	k'	α	R_s	Mobile phase[b]	R_s[c]
norleucine	5.27	1.10	1.01	30:70	2.30
aspartic acid	5.00	1.11	0.90	30:70	--
serine	2.50	1.09	0.80	30:70	0.43
leucine	4.93	1.14	1.30	30:70	--
valine	3.13	1.10	1.10	30:70	2.10
noevaline	3.20	1.08	0.74	30:70	0.83
glutamic acid	3.60	1.08	0.75	30:70	--
methionine	2.97	1.07	0.75	30:70	0.70
threonine	2.16	1.13	1.20	30:70	2.00
phenylalanine	6.27	1.08	0.80	30:70	1.10

a. The capacity factor of the first eluted enantiomer.
b. The numbers reprensent the volume ratio of methanol to TEAA buffer (0.3% TEAA, pH=6.2).
c. Data from: W. L. Hinze and D. W. Armstrong, Anal. Chem. 57(1985), 237-242.

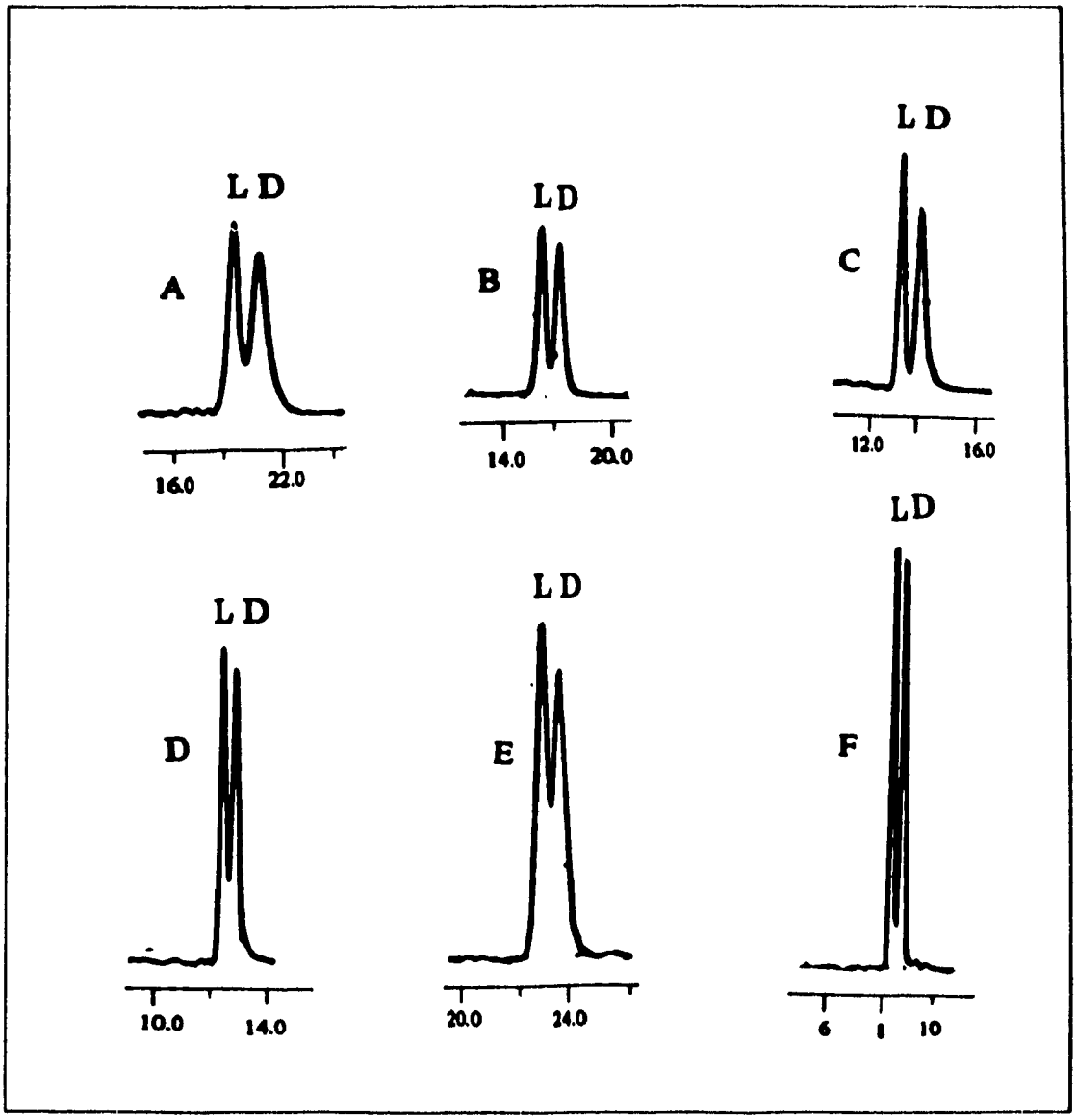


Figure 6.6. Chromatograms for the resolution of dansyl-DL-amino acids. (A) dansyl-DL -norleucine; (B) dansyl-DL-valine; (C) dansyl-DL-leucine; (D) dansyl-DL-aspartic acid; (E) dansyl-DL-glutamic acid; (F) dansyl-DL-threonine. Column, (S)-(-)-α-methylbenzylamine modified ß-cyclodextrin-bonded column (250 x 4.6 mm); mobile phase, 35:65 methanol/TEAA buffer solution (0.3% TEAA, pH = 5.5).

6.3.4.2. Enantiomeric Separations of DNP-amino acids

Dinitrophenyl amino acids (DNP-amino acids) have also been used for the quantitative amino acid estimation and the structural investigation of proteins since they were first applied by Sanger [17] to the determination of the N-terminal residues of insulin. The peptides or proteins react with fluoro-2,4-dinitrobenzene in alkaline solution to give DNP derivatives. On hydrolysis the peptide chain is broken to free amino acids but the original N-terminal amino acid remains largely in the form of its DNP derivatives which can then be extracted with ether and fractioned for identification [18]. Like the dansyl amino acids, DNP amino acid derivatives are also important in the protein chemistry.

Table 6.4 lists the resolution data for the enantiomers of DNP-amino acids. Some typical chromatograms are shown in Figure 6.7. In these cases, the D-enantiomers are eluted first.

6.3.4.3. Enantiomeric separation of some other chiral compounds

It was also found that the modified ß-cyclodextrin stationary phase exhibited a very good enantioselectivity for certain aromatic amino acids and other chiral compounds which contain only one benzyl ring in their molecules. The enantiomers of these chiral compounds, including phenylalanine, phthaloylalanine, tyrosine, carboxylic acids, and some pharmaceuticals, were separated on the methylbenzylamine and naphthylethylamine modified ß-cyclodextrin columns with methanol/TEAA buffer (0.3% TEAA) as the mobile phase. To date there have been no reports on the resolution of any enantiomers of these chiral compounds on a unmodified ß-cyclodextrin column.

Table 6.5 shows the optical resolution data for some aromatic amino acids which have only one benzene ring in the molecule. As can been seen, good resolution has been achieved for these D, L pairs with R_S values as large as 2.80 for phthaloylalanine.

Table 6.6 summarizes the optical resolution data for other chiral compounds. Most of them are carboxylic acids with only one benzene ring in the molecule. Very good resolutions have been achieved on the methylbenzylamine modified stationary phase with R_S values greater than 3.00 in several cases.

Table 6.7 shows the optical resolution data for chiral phenothiazine drugs on a naphthylethylamine modified ß-cyclodextrin-bonded phase column. Although the enantiomers of these compounds can be separated on the unmodified ß-cyclodextrin column, the resolution factors are much lower than these values.

Figure 6.8 shows some typical chromatograms of the chiral compounds.

To date, the chiral separations of a total of 57 chiral solutes has been tested on the modified and unmodified ß-cyclodextrin stationary phases. The 57 solutes involve a wide range of compounds. It has been found that the modified ß-cyclodextrin stationary phases have a much broader range of application than the unmodified stationary, especially when the modifier is methylbenzylamine. The percentage of chiral separated compounds increases about 25% with this modified phase.

Table 6.4.
Optical resolution of the enantiomers of DNP amino acids

SOLUTES [a]	β-CD–NH–CH(CH$_3$)– 				β-CD
	k' [b]	α	R_s	mobile phase [c]	R_s [d]
DNP-DL-α-amino-n-butyrc acid CH_3CH_2-CHCOOH NHR	3.20	1.05	0.70	30:70	0.60
DNP-DL-Norvaline $CH_3CH_2CH_2$-CHCOOH NHR	3.87	1.12	1.20	30:70	0.80
DNP-DL-Norleucine $CH_3(CH_2)_3$-CHCOOH NHR	6.35	1.13	1.83	30:70	2.45
DNP-DL-α-amino-n-caprylic acid $CH_3(CH_2)_5$-CHCOOH NHR	11.01	1.21	1.62	30:70	3.40
DNP-DL-methionine sulfone $CH_3SO_2CH_2CH_2$-CHCOOH NHR	1.67	1.08	0.90	30:70	0.90
DNP-DL-methionine $CH_3SCH_2CH_2$-CHCOOH NHR	3.42	1.17	2.57	25:75	1.50
DNP-DL-ethionine $CH_3CH_2SCH_2CH_2$–CHCOOH NHR	7.22	1.17	2.37	30:70	2.50
DNP-DL-citrulline $H_2NCONH(CH_2)_3$ CHCOOH NHR	1.75	1.07	0.80	30:70	0.80
DNP-DL-glutamic acid $HOOCCH_2CH_2$ CHCOOH NHR	10.20	1.06	0.70	30:70	0.90

a. R in the structures represents 2,4-dinitrophenyl.

b. The capacity factor of the first eluted enantiomers.

c. The number represents the volume ratio of methanol to TEAA buffer (0.3% TEAA, pH 5.5)

d. Data from: S. Li and W. C. Purdy, J. Chromatogr., 543(1991) 105.

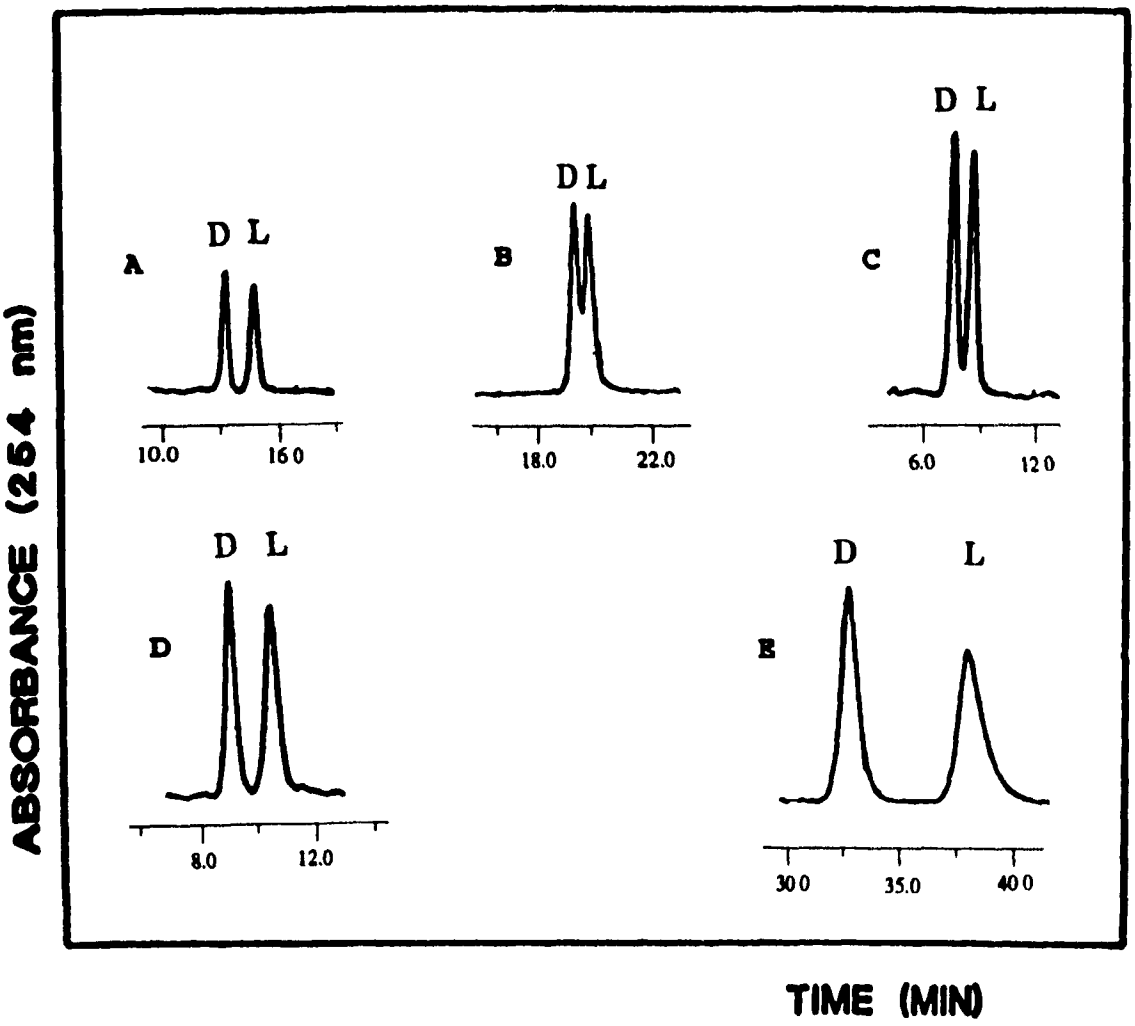
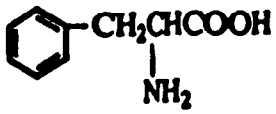
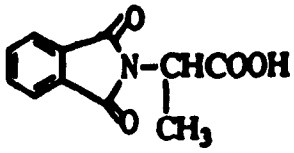


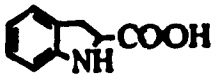
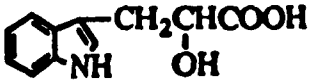
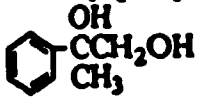
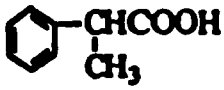
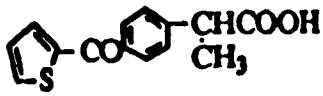
Figure 6.7. Chromatograms for the resolution of DNP-DL-amino acids. (A) DNP-DL-ethionine; (B) DNP-DL-glutamic acid; (C) DNP-DL-methionine; (D) DNP-DL-norleucine; (E) DNP-DL-α-amino-n-caprylic acid. Column, (S)-(-)-α-methylbenzylamine modified ß-cyclodextrin-bonded column (250 x 4.6 mm); the mobile phase composition is given in Table 6.4.

Table 6.5.
Optical resolution of the enantiomers of other amino acids

Solutes	β-CD–NH–CH(CH$_3$)–C$_6$H$_5$ k'[a]	α	R_s	Mobile phase
3-Indoletactylaspartic acid 	11.1	1.18	1.36	20% methanol 0.3% TEAA pH = 7.0
Phenylalanine 	2.06	1.17	1.50	20% methanol 0.3% TEAA pH = 5.5
Carbobenzyloxyalanine 	3.58	1.09	0.75	30% methanol 0.3% TEAA pH = 5.5
N-Phthaloylalanine 	1.45	2.56	2.80	50% methanol 0.3% TEAA pH = 5.5

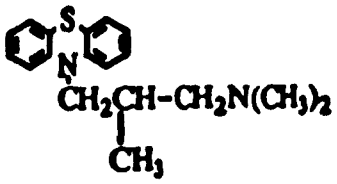
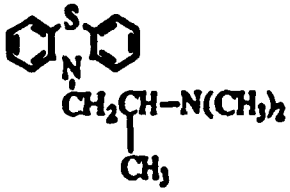
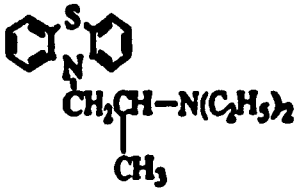
a. The capacity factor of the first eluted enantiomer.

Table 6.6.
Optical resolution of the enantiomers of chiral compounds

Solutes	β-CD–NH–CH(CH$_3$)–  k'[a]	α	R_s	Mobile phase
1. Indoline-2-carboxylic acid 	1.84	2.08	3.0	20% methanol 0.25% TEAA pH = 7.0
2. 3-Indoletactic acid 	11.1	1.18	1.36	25% methanol 0.25% TEAA pH = 6.2
3. 4-Methoxymandelic acid 	3.77	1.27	1.01	25% methanol 0.25% TEAA pH = 6.2
4. Mandelic acid 	0.42	2.54	3.33	30% methanol 0.3% TEAA pH = 5.5
5. 2-Phenylpropanediol 	1.0	1.19	1.01	30% methanol 0.3% TEAA pH = 5.5
6. 2-Phenylpropionic acid 	1.65	1.24	1.26	30% methanol 0.3% TEAA pH = 5.5
7. Suprofen 	5.25	1.11	0.90	40% methanol 0.25% TEAA pH = 6.0

a. The capacity factor of the first eluted enantiomer.

Table 6.7.
Optical resolution of the enantiomers of phenothiazines

Solutes	β-CD–NH–CH(CH$_3$)–Ar: k'^a	α	R_s	Mobile phase
1. Trimeperazine 	7.77	1.14	1.21	30% methanol 0.7% TEAA pH = 5.5
2. Promethazine 	7.74	2.40	3.67	30% methanol 0.3% TEAA pH = 5.5
3. Ethopropazine 	10.1	2.79	4.00	30% methanol 0.3% TEAA pH = 5.5

a. The capacity factor of the first eluted enantiomer.

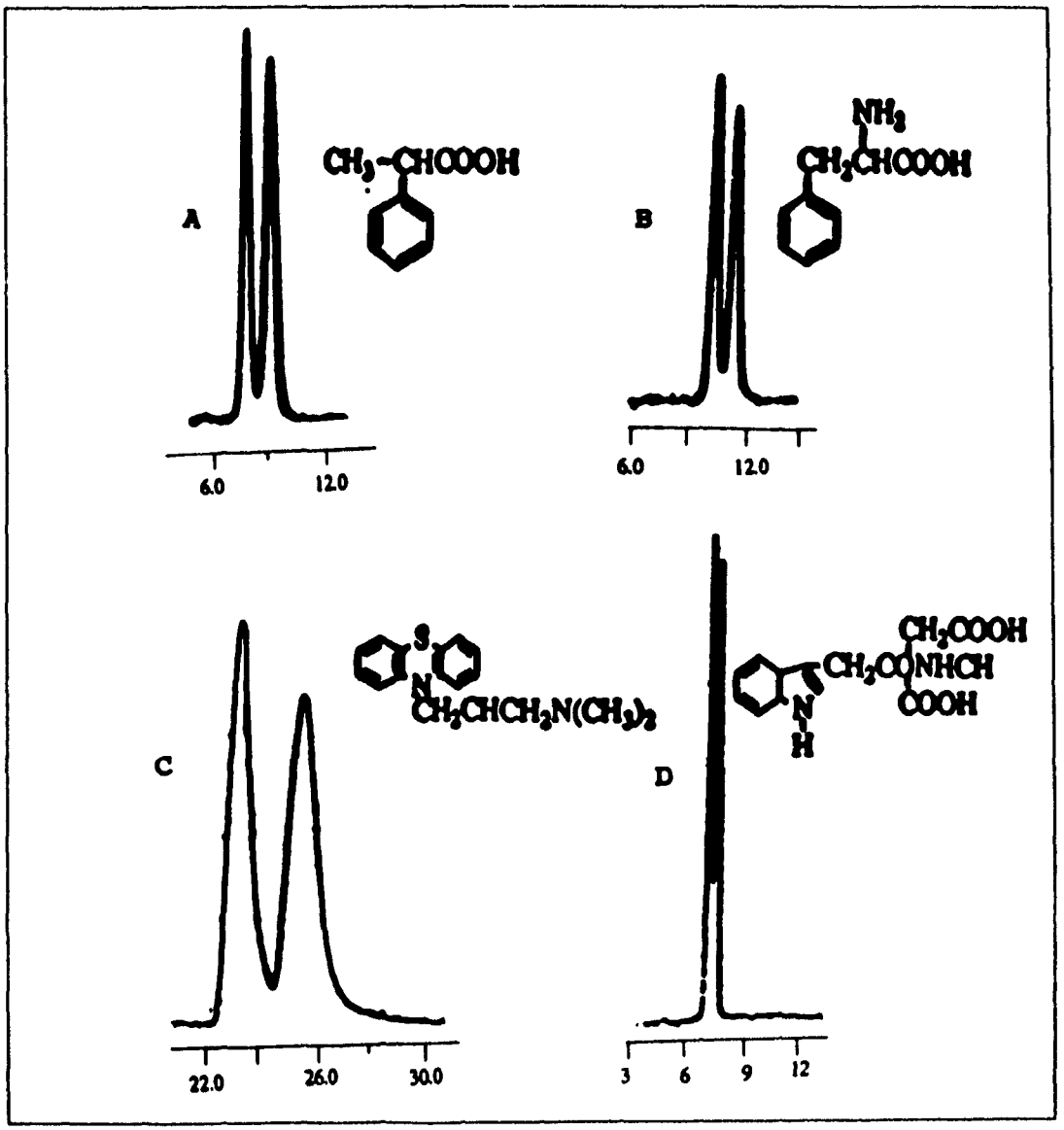


Figure 6.8. Chromatograms for the enantiomeric separation of some other chiral compounds. (A) 2-phenylpropionic acid; (B) phenylalanine; (C) trimeperazine; (D) indoletactylaspartic acid. Column, (S)-(-)-α-methylbenzylamine modified ß-cyclodextrin-bonded column (250 x 4.6 mm); mobile phase, 30:70 methanol/TEAA buffer (0.3% TEAA, pH 5.5).

6.3.5. Effect of mobile phase composition on the retention and resolutions

In this work, several polar solvents, such as water, methanol, ethanol, acetonitrile and tetrahydrofuran, were investigated as the potential mobile phase for the these modified ß-cyclodextrin stationary phases. It was found that most chiral compounds of interest cannot be eluted within a reasonable time from the modified ß-cyclodextrin columns using water alone as the mobile phase. The use of 100% methanol, ethanol, acetonitrile or tetrahydrofuran give no resolution at all. The methanol-water and ethanol-water mobile phases were found to provide much better selectivity than acetonitrile-water and tetrahydrofuran water mobile phases. The methanol-water system was chosen as the mobile phase.

In order to find the optimum conditions for good resolution, the effects of methanol concentration, TEAA buffer, and pH on the retention time and resolution were studied.

6.3.5.1. Effect of methanol concentration in the mobile phase

The effect of methanol concentration in the mobile phase on the retention and resolution was investigated by changing the methanol-water ratio in the mobile phase from 10:89.5 to 60:39.5 (v/v). TEAA concentration was 0.5%, and the pH was controlled at 5.5. It was found that the effect of methanol content on the retention and optical resolution gave almost the same tendencies as those observed on the unmodified ß-CD column. An increase in the methanol concentration results in a decrease in both retention time and resolution factor (see Figure 6.9). The effect of methanol concentration on the resolution is almost linear. When the methanol concentration reaches 75%, no resoltuion can be observed for almost all the chiral compounds.

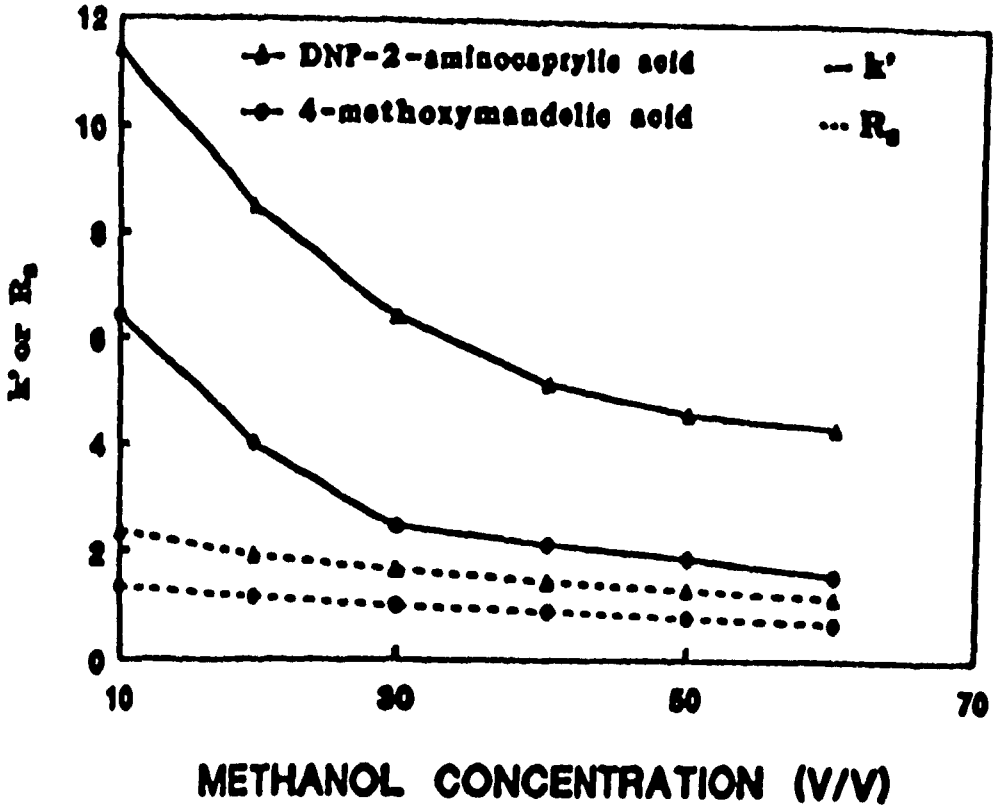


Figure 6.9. Effect of methanol concentration on the retention and resolution. Column, (S)-(-)-α-methylbenzylamine modified ß-cyclodextrin-bonded column; mobile phase, methanol-TEAA buffer (0.5% TEAA, pH = 5.5); flow rate, 1 mL/min.

6.3.5.2. Effect of TEAA concentration in the mobile phase

Figure 6.10 shows the effect of TEAA buffer concentration in the mobile phase on the retention and optical resolution. It was found that an increase in the TEAA concentration in the mobile phase results in a decreased retention time for both enantiomers. As was observed on unmodified ß-cyclodextrin stationary phase (see Chapter 4), the effect of TEAA concentration on the optical resolution is somewhat more complex. When the TEAA concentration

changed from 0% to 1.0%, two types of behaviour can be observed. At the beginning, the resolution increases with the increasing TEAA concentration and then it decreases when TEAA exceeds 0.3%. Resolution maxima are observed at a TEAA concentration of about 0.3%. These observations can be explained by considering both the effect of TEAA on the column efficiency (N) and enantioselectivity (α) and the relationship between resolution (R_S), α and N, as discussed in Section 4.3.4. When TEAA concentration exceeds 2.5%, no optical resolution can be observed for most of the chiral compounds.

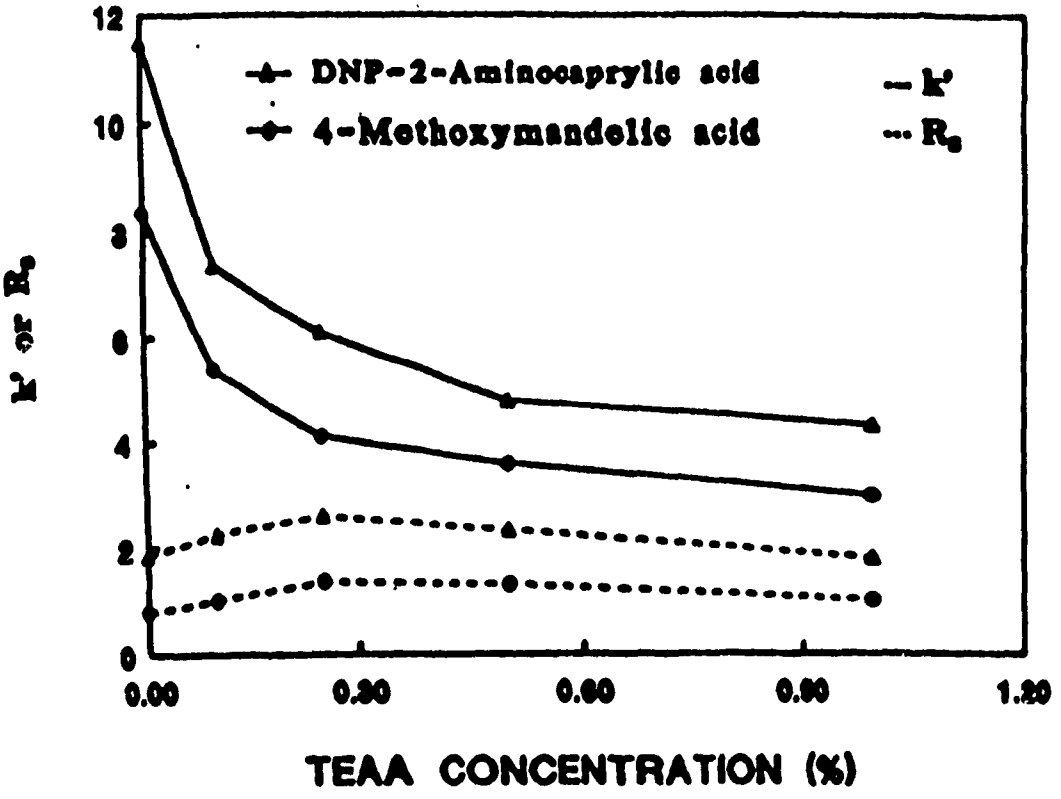


Figure 6.10. Effect of TEAA concentration on the retention and resolution. Column, (S)-(-)-α-mehtylbenzylamine modified ß-cyclodextrin-bonded column; mobile phase, methanol-TEAA buffer (35% methanol, pH = 5.5); flow rate, 1 mL/min.

6.3.5.3. Effect of pH

The effect of pH on the retention and resolution was investigated by changing pH of the mobile phase from 4.0 to 7.0 using a 0.5% TEAA buffer. Two typical plots are shown in Figure 6.11. It was found that pH only affects the retention and resolution of those compounds which have an ionization constant in the range of 10^{-4} to 10^{-7}. For example, over this pH range the compound trimeperazine, which has a pK_a value of 9.0, will be in its molecular form. Its retention and resolution is not affected by changing pH. The other line shows the effect of pH on dansyl-serine. Both the retention time and resolution decreased with increasing pH. This is probably due to ionization of this compounds.

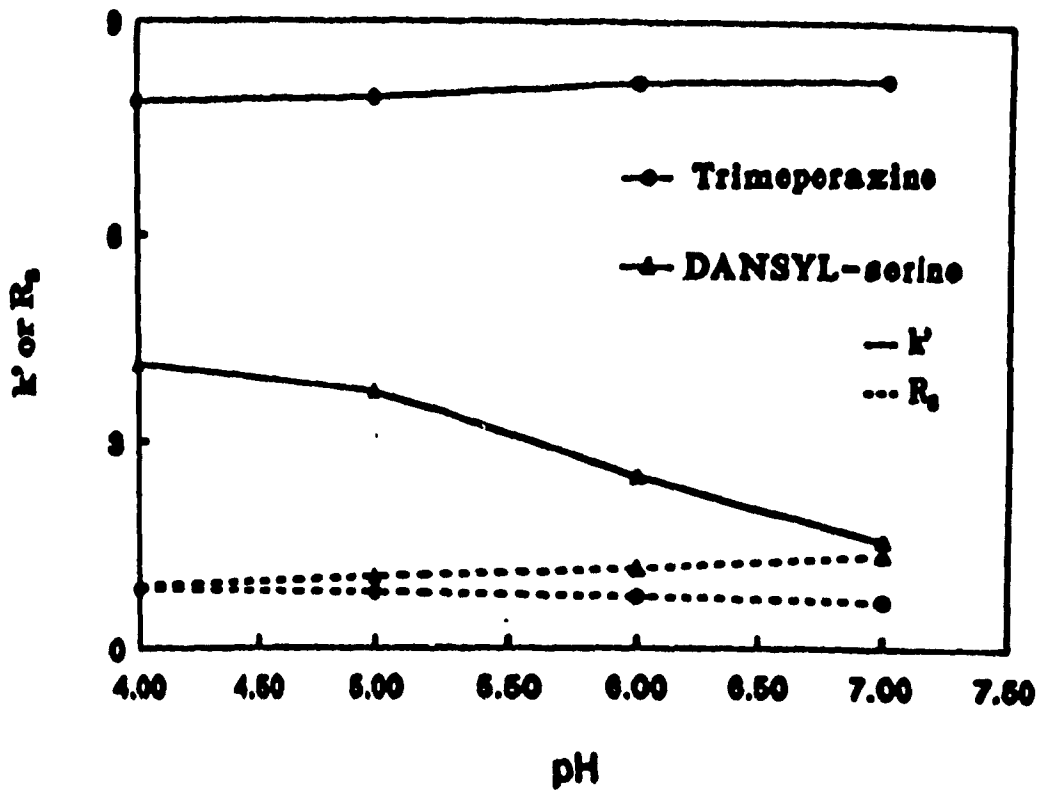


Figure 6.11. Effect of pH on the retention and resolution. Column, (S)-(-)-α-methylbenzylamine modified β-cyclodextrin-bonded column; mobile phase, methanol-TEAA buffer (0.3% TEAA, 35% methanol); flow rate, 1 ml/min.

In general, the effect of mobile phase composition can be summed up as follows: (i) increasing methanol content decreases the retention time and the resolution; (ii) the TEAA buffer improves column efficiency, but decreases the enantioselectivity; and (iii) neutral molecules are more readily retained than charged molecules.

6.4. CONCLUSIONS

These modified ß-cyclodextrin chiral stationary phases can be seen as a combination of donor-acceptor phase with cyclodextrin phase. They have, on the molecular level, an hydrophobic cavity, capable of forming inclusion complex with the hydrophobic moiety of the solute molecule; aromatic groups, capable of π-π (charge-transfer) interaction; polar hydrogen-bonding sites, which can form hydrogen-bonding with the polar functional groups of the solute; and bulky non-polar groups, which provide steric repulsion, van der Waals interaction and confirmational control. These multiple-interaction chiral stationary phases have exhibited a higher stereoselectivity toward a wide variety of chiral compounds. Enantiomeric separations of some chiral compounds which have only one benzyl ring in the molecule have been achieved without derivatization. These phases can be operated in the reverse-phase mode with mobile phase containing water and organic modifier. They are chemically and physically robust, and they have a similar chromatographic behaviour to the unmodified ß-CD column.

These multiple-interaction stationary phases have some disadvantages. (1) For some solutes the retention time is much longer than that with the unmodified ß-cyclodextrin stationary phase. This may be a problem for fast analyses. (2) The stationary phases have a light brown coloration probably

resulting from the formation of the nitroxide. The presence of the brown color makes the stationary phase unsuitable for thin-layer chromatography. (3) The lifetime of these stationary phases is shorter than that of unmodified ß-cyclodextrin stationary phase, especially when high acidity mobile phases are used.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

In this work the following contributions to original knowledge are claimed.

1. Liquid chromatographic behaviours of phenothiazines and their structurally-related compounds on the ß-cyclodextrin stationary phase were investigated. The possible retention mechanism was proposed.

2. Sixteen phenothiazines and structurally-related drugs were separated by isocratic and gradient-elution methods using a ß-cyclodextrin-bonded column.

3. Twelve pairs of amino acid enantiomers were separated as their dinitrophenyl derivatives using a ß-cyclodextrin-bonded phase column. The effects of pH, methanol and triethylammonium acetate buffer concentration in the mobile phase, and the structural features of the solutes on the retention and enantioselectivity were studied.

4. The chiral recognition mechanism of ß-cyclodextrin was studied by UV-visible, circular dichroism, and NMR spectroscopic methods using D- and L-enantiomers of DNP-amino acids as the model solutes. Such direct spectroscopic observation of the 'soluble models' of the chromatographic system' give detailed information about the stability and structures of the inclusion complexes and the chiral recognition mechanism.

5. A multiple-interaction type of chiral stationary phases was developed by bonding ß-cyclodextrin to silica gel and modifying the cyclodextrin cavity by flexibly capping its primary hydroxyl or small side. These modified ß-cyclodextrin stationary phases have demonstrated a high stereoselectivity toward a wide variety of chiral compounds.

6. The enantiomers of those chiral compounds, including amino acids and their derivatives, carboxylic acids, phenothiazine drugs etc., were separated using the modified ß-cyclodextrin stationary phases. The effect of mobile phase composition on the retention and enantiomeric resolution were studied.

Appendix A

Determination of Dissociation Constants by UV-visible Spectrophotometric methods

Dissociation constants for the inclusion complexes can be measured by observing the absorbance changes of guest molecules (G) as a function of added ß-cyclodextrin (H). Consider the equilibrium expression for the dissociation process:

$$H\text{-}G \rightleftharpoons H + G \qquad (1)$$

$$K_d = [H][G]/[C] \qquad (2)$$

$$[H_0] = [H] + [C] \qquad (3)$$

$$[G_0] = [G] + [C] \qquad (4)$$

$$K_d = ([H_0] - [C])([G_0] - [C])/[C] \qquad (5)$$

where $[H_0]$ and $[G_0]$ are the initial concentration of host and guest, respectively; [H] and [G] are the free concentration of host and guest, respectively. [C] is the equilibrium concentration of complex (H-G), and can be given by

$$[C] = \Delta Abs/\Delta\varepsilon \qquad (6)$$

where ΔAbs is the absorbance change, and $\Delta\varepsilon = \varepsilon_c - \varepsilon_h - \varepsilon_g$, ε_c, ε_h and ε_g are molar absorptivity of complex, host and guest, respectively.

Equation 5 is now rearranged to:

$$K_d\{C] = [C]^2 - [H_0]\{C] - [G_0]\{C] + [H_0]\{G_0] \quad (7)$$

Assuming that $[H_0]\{G_0] >> [C]^2$, equation (7) can be transformed to:

$$K_d\{C] = [H_0]\{G_0] - [C]\{G_0] - [C]\{H_0] \quad (8)$$

On combining (6) and (8) the following equation is obtained

$$[H_0]\{G_0]/\Delta Abs = K_d/\Delta\varepsilon + ([G_0] + [H_0])/\Delta\varepsilon$$

Since $\Delta\varepsilon$ and K_d are constants this equation represents a straight line when $[H_0][G_0]/\Delta Abs$ is plotted against the sum of the stoichiometric concentrations ($[G_0] + [H_0]$).

Appendix B

Determination of Dissociation Constants by Nuclear Magnetic Resonance Spectroscopic method

In NMR method, the dissociation constants are determined by measuring the changes in chemical shift of host (guest) protons as a function of added guest (host). In our measurements, the dissociation constants were determined by measuring the chemical shift changes for H3 resonance of ß-cyclodextrin as a function of added DNP-amino acids. Consider the equilibrium expression for the dissociation process

$$H\text{-}G \rightleftharpoons H + G \tag{1}$$

$$K_d = [H][G]/[C] \tag{2}$$

$$[H_0] = [H] + [C] \tag{3}$$

$$[G_0] = [G] + [C] \tag{4}$$

$$K_d = ([H_0] - [C])([G_0] - [C])/[C] \tag{5}$$

where all symbols have the same mean as in Appendix A. As point out, the system is in NMR chemical-shift fast-exchange limit. The observed chemical shift can be given by:

$$\delta_{obsd} = [H]\cdot\delta_H/[H_0] + [C]\cdot\delta_C/[H_0] \tag{6}$$

where δ_{obsd} is the observed chemical shift for ß-cyclodextrin proton (H3) in the presence of DNP-amino acid in the solution. δ_H is the chemical shift for the proton H3 of free ß-cyclodextrin, and δ_C is the chemical shift for the proton H3 of ß-cyclodextrin which have a guest molecule in its

cavity.

Combining (3) and (6) produces:

$$\delta_{obsd} = \delta_H([H_0] - [C])/[H_0] + [C]\delta_C/[H_0] \quad (7)$$

The change in chemical shift is given by:

$$\Delta\delta = \delta_{obsd} - \delta_H \quad (8)$$

Combining (7) and (8) gives:

$$\Delta\delta = (\delta_H - [C]\delta_H/[H_0] + [C]\delta_C/[H_0]) - \delta_H \quad (9)$$

which on rearrangement transforms to:

$$\Delta\delta = [C](\delta_C - \delta_H)/[H_0] \quad (10)$$

Letting $Q = \delta_C - \delta_H$, [C] can be express as:

$$\Delta\delta[H_0]/Q = [C] \quad (11)$$

Equation (5) is now rearranged to:

$$[C]K_d = [C]^2 - [H_0][C] - [G_0][C] + [H_0][G_0] \quad (12)$$

Assuming that $[H_0][G_0] >> [C]^2$, equation (12) cam be transformed to:

$$K_d[C] = [H_0][G_0] - [G_0][C] - [H_0][C] \quad (13)$$

Subtituting (11) to (13) produces the following equation:

$$[G_o]/\Delta\delta = K_d/Q + ([G_o] + [H_o])/Q \qquad (14)$$

Thus by plotting the $[G_o]/\Delta\delta$ versus $[G_o] + [H_o]$ a straight line is obtained with intercept K_d/Q and a slope $1/Q$. Therefore,

$$K_d = \text{intercept/slope} \qquad (15).$$