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Enantiomeric separation in pharmaceutical analysis: A chromatographic approach

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ABSTRACT

Chirality plays a major role in biological processes, and the enantiomers of a bioactive molecule often possess different biological effects. For example, all pharmacological activity may reside in one enantiomer of a molecule, or enantiomers may have identical qualitative and quantitative pharmacological activity. In some cases, enantiomers may have qualitatively similar pharmacological activity, but different quantitative potencies. Since drugs that are produced by chemical synthesis are usually a mixture of enantiomers, there is a need to quantify the level of the isomeric impurity in the active pharmaceutical ingredient. Accurate assessment of the enantiomeric purity of substances is critical because isomeric impurities may have unwanted toxicological, pharmacological, or other effects. Such impurities may be carried through a synthesis and preferentially react at one or more steps and yield an undesirable level of another impurity. The determination of a trace enantiomeric impurity in a sample of a single enantiomer drug substance in the presence of a range of other structurally related impurities and a large excess of the major enantiomer remains challenging. The development of chiral resolution methods is considered an urgent need in pharmaceutical, agricultural, and other chemical industries. Perhaps most essential is the development of new chiral drugs, which are necessary because of the different physiological properties of enantiomers. Various approaches to chiral resolution have been developed and used, but the direct resolution by liquid chromatography on chiral stationary phases has proven to be one of the most effective, practical, and economical. This volume deals with the art of chiral resolution by liquid chromatography, focusing on high-performance liquid chromatography (HPLC), sub and supercritical fluid chromatography (SFC), capillary electrochromatography (CEC), and thin-layer chromatography (TLC). These methods are examined as they are used in analysis and development of drugs, pharmaceuticals, xenobiotics, and other chiral molecules.

Key words: Chirality, Enantiomer, Chromatography, Complexing agent, Chiral selector.

INTRODUCTION

Stereochemical analysis has become an increasingly important problem in the pharmaceutical field, since numerous pharmacologically active agents are chiral and their two enantiomeric forms usually exhibit different physiological properties. Numerous examples exist where the two enantiomeric forms manifest different pharmacological actions, potencies, biodistribution and disposition kinetics, or host toxicities. For instance, most of the β-blocking activity of β-blockers such as propranolol and metoprolol is attributed to the (S)-enantiomer[1]. Furthermore, stereoselective differences have also been observed in the metabolism and pharmacokinetics of the two enantiomers of the above mentioned compounds. Traditional methods for determining the enantiomeric composition of chiral compounds, such as optical rotation measurement or fractional recrystallization of diastereomeric salts, are usually difficult, insensitive, inaccurate, and limited in applicability. Hence modern chromatography, because of its reproducibility, accuracy, selectivity, sensitivity, and speed, has become the technique of choice for the analysis of enantiomers. Since most of the recent developments and applications in enantiomeric resolution have been performed using liquid chromatography because of its wider applicability, particularly in the pharmaceutical field. There are three different approaches under which chromatographic resolution of enantiomers can be achieved[2].

1. The first approach involves the precolumn conversion of the enantiomers to diastereomers by reaction with a chiral derivatizing agent. The resulting diastereomers are then resolved on an achiral stationary phase using an achiral mobile phase.
2. The second approach involves the direct resolution of enantiomers on an achiral stationary phase using a mobile phase that contains a chiral component.
3. The third approach, which recently has received a great deal of research and development, involves the direct resolution of enantiomers using selective chiral stationary phases.

In these methods, the chemistry, applicability, and limitations of the three different liquid chromatographic techniques for the resolution of enantiomers are examined. Particular emphasis are given to the chromatographic approach, in which chiral stationary phases are utilized, since this area has recently enjoyed numerous interesting developments[3].

Method

1. Indirect liquid chromatographic resolution of enantiomers via precolumn formation of diastereomeric derivatives: Unlike enantiomers, whose physical properties are identical, diastereomers may have very different properties, such as solubility and polarity that permit them to be easily resolved by ordinary chromatographic means. Therefore, by converting enantiomers to diastereomers via chemical reactions with chiral derivatizing reagents, their chromatographic separation can be easily achieved. Oftentimes, the most difficult task in this chromatographic approach involves the selection of a suitable chiral reagent rather than the development of a chromatographic procedure for separating the derivatized diastereomers. Indeed, there are several factors involved in selecting a chiral reagent that can affect the applicability, accuracy, and resolvability of the chromatographic procedure[4].

1. The chiral reagent should be stable and easy to prepare or commercially available in a state of high optical purity. This is important since the degree of optical purity of the chiral reagent has a direct influence on the accuracy and the maximum detectable optical purity of the enantiomeric solutes [5].
2. The reaction should be mild, so that no racemization of the chiral center of the solutes and reagent occurs.
3. The derivatization must be quantitative and complete. Otherwise, determination of the enantiomeric purity could be inaccurate, since the reaction of enantiomers with a chiral substrate can occur at different rates [6].
4. The chiral solute should contain only one functional group for derivatization.
5. To enhance the detectability of the derivatives, the chiral derivatizing reagent should contain a chromophore or fluorophore.
6. Furthermore, since the chromatographic resolvability of the derivatized diastereomers is dependent on the degree of difference in various properties possessed by them, such as polarity, energies of adsorption, the accessibility of various polar substituents, and molecular structure [7]. A derivatizing reagent that can maximize the above differences should be selected, indeed, it has been demonstrated that the chromatographic resolution is greatly enhanced by the following factors: (a) The conformational ridigity of the groups attached to the chiral center of the derivatizing reagent, (b) The short distance between the two chiral centers of the derivatized diastereomers, and (c) The large size differential in the groups attached to the chiral reagent [8].
7.

Applications: Recently reported chiral derivatizing reagents and their chromatographic applications for the analysis of enatiomeric drugs are listed in Table 1. Various chiral reagents like isothiocyanate, isocyanate, acylchloride, and anhydride derivatives were used to derivatize p-adrenergic blocking drugs containing an aminol alcohol functional group, while several chiral amines were used to derivatize nonsterodial antiinflamatory drugs containing a 2-aryl propionic acid functional group.

Table 1: Chiral derivatizing agents and their applications

Chiral Reagents	Functional group Reacted To	Separation Mode	Application
2,3,4,6-tetra-*O*-acetyl-ß-*D*-glucopyranosyl isothiocynate	Amine	*Reverse phase*	Catecholamins, ß-blockers.
S(-)-1-phenylethyl isocynate	Amine	*Reverse phase*	Propranolol, Oxprenolol
(+)and(-)-α-methyl-1-naphthaleneacetic acid	Amine	*Normal phase*	DOPA,Amino acids,Ornithrine

Several of the reagents listed exhibited remarkable generality in resolving enantiomers having similar functional groups. The use of 2,3,4,6-tetra-O-acetyl-p-D-glucopyranosyl isothiocyanate and the ortho,ortho derivatives of *(R, R)* tartaric acid anhydrides brought about the enantiomeric resolution of a wide range of amino alcohols [9]. Nevertheless, chiral derivatization of compounds of other classes still have to be investigated on a case by case basis. This and the unavailability of several chiral derivatizing reagents listed with high optical purity severely limit the use of this chromatographic approach for the analysis of enantiomers[10].

2.Chromatographic resolution of enantiomers using chiral mobile phase: This chromatographic approach, which requires no tedious precolumn derivatization of the sample and no specialized chiral stationary phase, involves the use of mobile phases containing novel chiral additives to resolve

enantiomers. Based on the type of chiral additives and the mechanism under which the enantioselectivity is achieved, this approach can be classified into three classes: Ligand exchange chromatography, Chiral ion-pair chromatography, and Chromatography using mobile phases containing macromolecular aggregates[11].

a. Ligand Exchange Chromatography The two general approaches in which ligand exchange chromatography is performed are illustrated in Figure 1. The first approach, pioneered by ***Davankov*** for the resolution of amino acid enantiomers, employed stationary phases consisting of amino acid ligands bonded to polymeric resins on which Cu (II) metal ions were subsequently loaded [12].

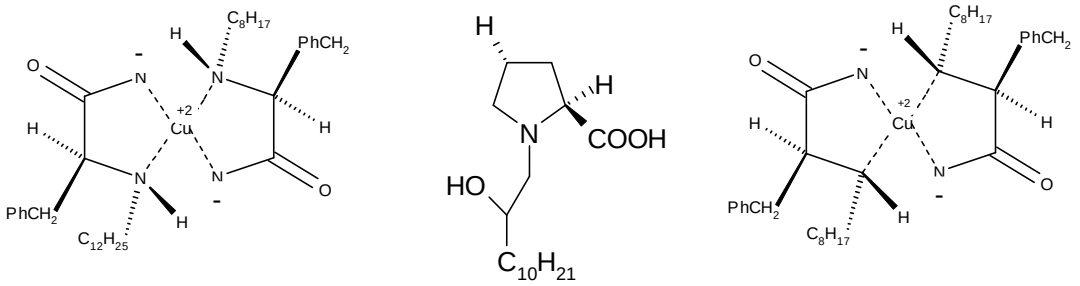

Figure 1: Chemical structure of legand exchangers

These polymeric sorbents, although having exhibited remarkable enantioselectivity for amino acids, were not stable to pressure and hence not applicable to HPLC. Consequently, a series of chiral ligands was bonded to silica gels; under either normal or reversed phase mode, this resulted in very efficient enantiomeric separation of amino acids and amino acid derivatives. To date, most of the applications using this approach have been centered on the enantiomeric analysis of amino acids, and no enantiomeric drug analysis has been reported [13].

Table 2: Chiral selector and Metal ion for Ligand Exchange Chromatography

Chiral Selector	Metal Ion	Stationary Phase	Solute Enantiomers
L- proline	Cu^{+2}	Silica gel	Thyroid harmone, α-Methyl DOPA,
L- proline	Cu^{+2}	C_{18}	α-Hydroxy acids
(R,R)-+-tartaric acid mono-n-octylamine	Cu^{+2}	C_{18}	Ethanol amine

The second approach, termed dynamic ligand exchange chromatoqraphy was pioneered in 1979 by ***Karger*** , ***Hare*** and ***Gil-Av*** for the resolution of amino acid enantiomers[14]. They showed that by adding chiral metal complexing agents to the mobile phases, very efficient separation of amino acid enantiomers and their derivatives can be achieved using conventional achiral stationary phases such as reversed phase or ion exchange packings. The mechanism of the resolution is based on the difference in the stabilities and therefore the equilibrium concentrations as well as the difference in the partitioning or adsorption behaviors of the two diastereomeric ternary complexes formed by the solute enantiomers and the chiral metal ligand[15]. Various dynamic ligand exchange chromatographic resolutions of non amino acid compounds are illustrated in Table 2. It should be noted that in most cases, the metal complexing agent consists of a certain optically pure Z,-amino acid or derivative and Cu (II). Copper (II) was the most frequently used transition metal, probably because of its strong chelating ability[16].

b. *Chiral ion pair chromatography*: Ion pair chromatography is a technique in which the retention of ionized compounds is regulated by the addition of a counter ion into the mobile phase. If the

counter ion is chiral, stereoselective separation can be achieved. The mechanism of the chiral separation is based on the difference in partitioning or adsorption behaviors of the diastereomeric ion pairs formed by the enantiomeric solutes and the chiral counter ion. Usually the binding forces consist of electrostatic and hydrogen bonding and mobile phases of low polarity are normally used to allow a satisfactory degree of ion pair formation. The stereoselectivity of the separation is dependent on the structure of the counter ion. The enantioselectivity of the separation is high when the counter ion contains binding groups having a rigid ring system with bulky groups in the vicinity of the chiral center[17]. Recent applications using this chromatographic technique are summarized in Table 3.

Table 3: Chiral Ion pair chromatography Chiral selector and Mobile phase.

Chiral selector	Enantiomer solute	Mobile phase	Stationary phase
Quinine	Tropic acid, Atropic acid, Naproxane	Dichloro methane: Pentanol	Diol
Camphorsulfonate	ß-Blocker	Dichloro methane: Pentanol	Diol
Tartaric acid ester	Ephidrine	(Dichloromethane: Pentanol)+Phosphate buffer	Phenyl

***c. Chromatography using mobile phases containing macromolecular aggregates*:** Natural macromolecules such as proteins and cyclodextrins have been known to exhibit stereoselective binding with low molecular weight molecules [18]. Consequently, when macromolecules are used as mobile phase additives, stereoselective separations can sometime be achieved. In general, the retention of the enantiomeric solutes as well as the selectivity of the separations is regulated by the concentration of the macromolecules. An increase in the concentration of the macromolecules usually decreases the retention of the solutes (by enhancing their partitioning to the mobile phase) and increases the selectivity of the separation [19]. The various stereoselective separations utilizing macromolecular additives reported in the literature are summarized in Table 4.

Table 4: Chromatography separation using mobile phases containing macromolecular aggregates

Mobile phase additives	Stationary phase	Enantiomer solute
Human serum albumin	C_{18}	Carboxylic acid
α-Cyclodextriin	C_{18}	Mephenytoin
ß-Cyclodextriin	C_{18}	Norgestrel
γ-Cyclodextriin	C_{18}	Mandelic acid

***3. Chromatographic resolution of enantiomers using chiral stationary phase*:** This chromatographic approach, have several advantages over the first two approaches. The use of chiral stationary phases not only permits the use of inexpensive ordinary mobile phases but also obviates the need to derivatize the solutes with chiral reagents (which may be impure, may be unavailable, or may exhibit different reaction rates with the enantiomers). The various chiral stationary phases reported can be classified as:

a. Pirkle type chiral stationary phases.
b. Cyclodextrin bonded stationary phases.
c. Protein bonded stationary phases.
d. Chiral polymer stationary phases.

a. *Pirkle type chiral stationary phases*: A series of chiral stationary phases capable of separating a large range of enantiomers was designed by ***Pirkle*** and coworkers based on a three-point chiral

recognization model proposed by ***Dalgliesli***. According to the model, at least three simultaneous interactions between the chiral stationary phase and the enantiomeric solute are required for chiral resolution.

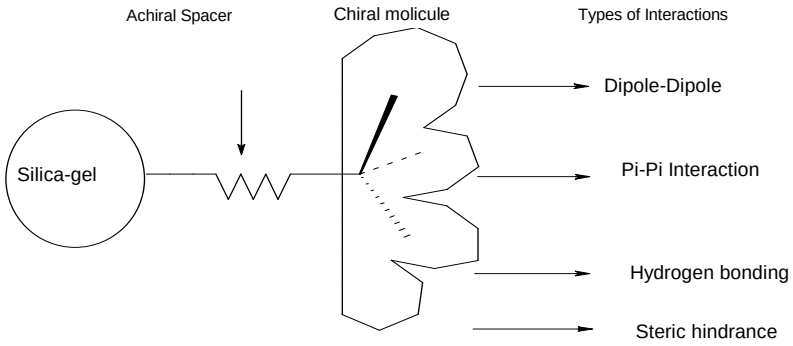

Figure 2: General structure of various bonded Pirkle type stationary phases

Furthermore, at least one of these interactions, attractive or repulsive, must be stereochemically controlled. The two general forms of these stationary phases, which differ by whether they are ionically or covalently bonded to the silica backbone, are illustrated in Figure 2. These stationary phases contain a number of possible sites for interactions[20]:

1. π-π bond donor-acceptor interaction from the 3,5 dinitrobenzoyl ring.
2. Hydrogen bonding from the amide hydrogen and carboxyl.
3. Dipole formed at the amide linkage.
4. Possible Van der Waals attractive π-π interaction or steric repulsion (due to the relative bulkiness of the group between the *R* group).

These sites provide a number of ways for the chiral stationary phase to interact with the enantiomeric solute. These enantiomeric solutes include a wide range of molecules[21]. A wide variety of Pirkle type stationary phases are now commercially available (Table 5). They can be classified as either π-electron acceptor or π -electron donor stationary phases. In general the π-electron acceptor stationary phases are used to resolve π-electron donors such as aromatic alcohols, aromatic amines, aromatic sulfoxides, hydantoins, succinimides, and lactams[22], while the π-electron donor stationary phases are employed to resolve amines, alcohols, amino acids, and amino alcohols that have been derivatized with a π-electron acceptor such as 3,5 dinitrobenzoyl chloride [23].

Table 5: Chromatography separation using Pirkle Type Chiral Stationary Phases

Pirkle Type Chiral Stationary Phases
(R)-N-DNB(***Dinitrobenzoyl***)-phenylglycine(Ionic)
(R)-N-DNB-phenylglycine(Covalent)
(S)-N-DNB-phenylglycine(Covalent)
(S)-N-DNB-leucine(Ionic)
(R)-N-DNB- leucine (Covalent)
(S)-N-DNB- leucine (Covalent)
(R)-(1-phenyl)ethylurea
(R)-naphthylalanine

The main advantage of the Pirkle type chiral stationary phases is their broad applicability in resolving a wide range of compounds. Furthermore, relatively high chromatographic efficiency and enantiomeric selectivity have been routinely observed in applications using these stationary phases. The major disadvantage of these chiral stationary phases is that enantiomers containing polar groups such as amino or carboxy functions usually have to be derivatized in order to obtain satisfactory enantiomeric resolution. Some of these stationary phases are not compatible with aqueous mobile phases and thus are of limited use in the analysis of drugs in biological fluids[24].

b. Cyclodextrin Bonded Stationary Phases: Chiral cyclodcxtrins are cyclic oligosaccharides consisting of six or more *D-(+)*-glycopyranose units. They have the shape of a hollow truncated cone with the larger end ringed with the secondary hydroxyl groups and the smaller end rimmed with primary hydroxyl groups (Figure 3a, 3b). The interior of the cavity, which contains glycosidic oxygen bridges, is, therefore, relatively hydrophobic[25]. The smallest cyclodextrin homologs, α-, ß- and γ-cyclodextrins, which consist of six, seven, and eight glucose units respectively, are commercially available. Although various cyclodextrin stationary phases have been synthesized by either cross-linking cyclodextrin polymers or chemically attaching cyclodextrins to silica via different bonding processes[26], only the cyclodextrin bonded stationary phases developed by ***Armstrong*** and coworkers are commercially available, These stationary phases, synthesized by chemically linking cyclodextrin to silica via a hydrolitically stable and non-nitrogen-containing linkage, have found widespread applications in resolving various optical, geometrical and structural isomers as well as other routine compounds.

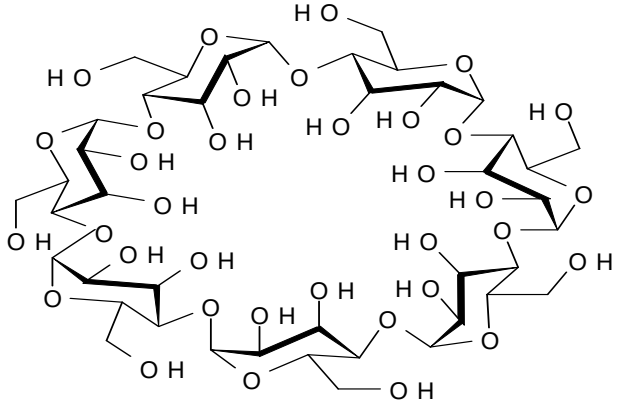

Fig3a. Cyclodextrin derivatives (α derivatives contains 6 glutamine units , β derivatives contains 7 glutamine units , γ derivatives contains 8 glutamine units)

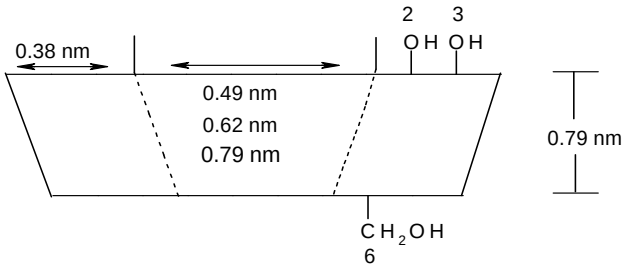

Figure3b: The chemical structures of Cyclodextrin and their derivatives (for α: 0.49 nm, for β: 0.62nm, for γ: 0.79nm)

The basic property of cyclodextrins allows to a numerous chemical separations is their ability to include selectively, in solution, a variety of guest molecules in their cavities. The formation of the inclusion complex may be caused by either a hydrophobic effect (due to favorable dipole-dipole interaction between the cyclodextrin and the guest molecule), hydrogen bonding (between the cyclodextrin's hydroxyl groups and the guest molecule), the release of high energy water or organic modifier during complex formation, and a combination of these factors [27]. The basis for chiral recognition by cyclodextrins can be rationalized by ***Dalgliesh's*** three-point interaction concept [28]. The three points of interaction consist of a dipole-dipole interaction with the cyclodextrin's cavity and two other interactions with the cyclodextrin's rim. The two interactions with the rim can be attractive hydrogen bondings or repulsive steric interactions. Various enantiomeric separations of pharmaceuticals using cyclodextrin bonded stationary phases are summarized in Table 6. Most of these separations were achieved using aqueous mobile phases and the ß-cyclodextrin bonded stationary phase, and without the need to do precolumn derivatization of the drugs.

Table 6: Chromatography separation using Cyclodextrin Bonded Stationary Phases

Stationary Phase	Applications
ß-Cyclodextriin	Propranolol, Metoprolol
Acetylated ß-Cyclodextriin	Norgestrel

c. Protein bonded stationary phase: The ability of proteins stereoselectivily to bind low molecular weight enantiomers has been observed in various protein-drug equilibria studies. This remarkable ability of proteins originates from their complex three-dimensional structures, which contain various functional groups capable of manifesting different types of interactions. This feature was used by two different research groups in designing their chiral stationary phases. One type of protein column was developed by ***Allenmark*** by covalently bonding bovine serum albumin to silica gel. Another type of protein bonded stationary phase was synthesized by ***Hermansson*** using ionic bonding and then cross-linking a-acid glycoprotein to silica gel. Both types of stationary phases have demonstrated unusual ability to resolve enantiomers [29]. The various enantiomeric separations using the two chiral stationary phases are summarized in Table 7. Even though both of these two stationary phases are now commercially available, to date the a-acid glycoprotein bonded stationary phase appears to offer a wider range of application to molecules of pharmacological interest. As in the case of cyclodextrin bonded stationary phases, many separations performed using these two stationary phases were achieved without the need to derivatization enantiomeric solutes. Furthermore, these columns allow the use of aqueous mobile phases, which are preferred for drugs in biological fluids. Also, this approach has the advantages of optimizing the solute retentions as well as the enantioselectivity by adjusting such separation parameters as ionic strength, pH, and ion pair reagents [30].

Table 7: Chromatography separation using Protein bonded stationary phase

Protein Bonded Stationary Phase
Bovine serum albumin
ß acid-glycoprotein
α- acid-glycoprotein

d. Chiral Polymer Stationary Phases: The chiral recognition ability of optically active polymers such as cellulose, cellulose derivatives, and polyamides has been known for some time. However, until recently, most enantiomeric separations using chiral polymers were performed

using planar or low pressure column chromatography. Part of the reason may be caused by the difficulty in preparing cross-linked polymers having sufficient mechanical strength, minimal swelling in various solvents, and appropriate size for high pressure liquid chromatography. Figure 4 represents Chemical immobilization pathways for cyclodextrins on silica gel**.**

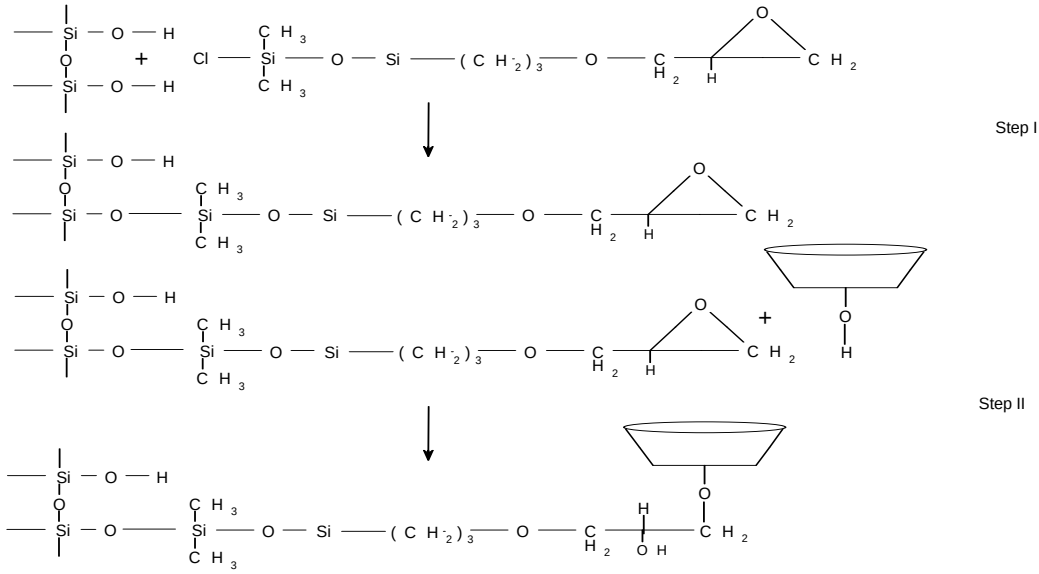

Figure 4: Chemical immobilization pathways for Cyclodextrin on silica gel

Consequently, simple alternative approaches for preparing chiral polymer stationary phases, which involve either physically coating or covalently bonding the chiral polymers to a macroporous support, have been developed. Indeed, macroporous silica coated with poly (triphenylmethyl methacrylate) and cellulose derivatives or covalently bonded with polyacrylamides have demonstrated remarkable ability in resolving various enantiomers. Recent enantiomeric separations using various chiral polymer stationary phases are summarized in Table 8. It should be noted that the coated polymer packings usually exhibited different selectivity from their analogous packings prepared by cross linking pure polymers. In the case of coated polymer stationary phases, only mobile phases consisting.of non-solvents of the chiral polymer can be used to prevent its leaching. There are controversies concerning the mechanism for chiral recognition by chiral polymers [31]. Nevertheless, it appears that chiral recognition does not result from the direct interactions between the individual optically active residue of the chiral polymers and the enantiomers but rather from the inclusion of the enantiomers into the asymmetric cavities of the three-dimensional polymeric network, Since several chiral polymeric stationary phases are now commercially available, numerous applications using this type of stationary phase will undoubtedly appear in the near future[32].

Table 8: Chromatography separation using chiral Polymer Stationary Phase

Stationary phase	Applications
Cellulose carbamate polymer	Mephobarbital,Ethotoine
Poly(acrylamide) polymer	Chlothalidone
Poly(methacrylamide) polymer	Thalidomide
Microcrystalline cellulose triacetate	Benzothiodiazepines

Future trends: The discussed method approache for chiral resolution have been proven to be one of the most effective, practical, and economical method for separation of enantiomers. The methods are useful for separation of different enantiomer as well as enantiommer and non enantiomer. These methods are are used in analysis and development of protein based drugs, chiral molecules, xenobiotics, and other pharmaceuticals.

Recent developments

Recent advances in enantiomer separations found by capillary electrophoresis (CE), using proteins as chiral selectors, affinity capillary electrophoresis (ACE) with free solutions and capillary electrochromatography (CEC) with protein immobilized capillaries. The new separation principle, recent advances in this field found with ACE. In ACE, various enantiomer separations reported using either plasma proteins or egg white ones. On the contrary, only a limited number of enantiomer separations have been successfully achieved in CEC. For further investigations, such as efficiency, durability and reproducibility of capillaries will be necessary for the use of routine analyses. The study of enantioselective drug–protein binding is important in pharmaceutical developments. Some applications including high-performance CE/frontal analysis (HPCE/FA), UPLC are introduced for recent development in enantiomeric analysis.

CONCLUSION

It is clear that the range of liquid chromatographic techniques for enantiomeric analysis is now very broad. This is because no single liquid chromatographic technique can provide superior and universal chiral separation of all classes of compounds. Consequently, chiral liquid chromatographic separation is not trivial, since it is still difficult to decide which technique would work for an unfamiliar chiral compound. Undoubtedly, further advances in the development of more universal chiral liquid chromatographic techniques and in the understanding of chiral recognition mechanisms are needed before chiral liquid chromatography can be considered routine.

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