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Review

High-performance liquid chromatographic enantioseparation of drugs containing multiple chiral centers on chiral stationary phases

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Received 16 February 2009; received in revised form 7 March 2009; accepted 15 March 2009

Abstract

In recent years there has been considerable interest in the synthesis and separation of enantiomers of organic compounds especially because of their importance in the biochemistry and pharmaceutical industry. High-performance liquid Chromatography is a very useful method for the direct separation of enantiomers. However, about 30–40 years ago, commercially available chiral stationary phases were very limited. Researchers developed many novel chiral stationary phases for gas and liquid chromatography, and found these phases were effective practically to the separation and analysis of various chiral compounds. Enantioseparation of drugs with multiple chiral centers is challenging. This review describes resolution of some drugs with multiple chiral centers using polysaccharide-type chiral stationary phases.

Keywords: Enantiomer separation; Drug; Chiral stationary phases; HPLC

1. Introduction

Enantiomers are two chemically identical molecular species which differ from each other as nonsuperposable mirror images [1]. The most simple and vivid model for enantiomeric structures is the two hands, left and right. Enantiomers, in addition to diastereomers and cis-trans-isomers, are thus a special case of stereoisomers. The chirality (handedness) of enantiomeric molecules is caused by the presence of one or more chirality elements (chirality axis, chirality plane, or chirality centre, e.g., asymmetric carbon atom) in their structure.

The chirality sense and optical activity of the enantiomers are determined by their absolute configuration, i.e., the spatial arrangement of the atoms in the molecule. In contrast to their conformation, the configuration of enantiomers cannot be changed without a change in the connectivity of constituent atoms. Designation of the configuration of enantiomers should be made in accordance with the Cahn-Ingold-Prelog R, S-system. The Delta-Lambda designations for enantiomers of octahedral complexes and the D, L Fischer-Rosanoff designations for amino acids and sugars are also in use.

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2. Chiral chromatography

The biological activity of chiral substances often depends upon their stereochemistry, since the living body is a highly chiral environment. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics. The importance of chirality of drugs has been increasingly recognized, and the consequences of using them as racemates or as enantiomers have been frequently discussed in the pharmaceutical literature during recent years.

Drugs that are derived from natural products are usually obtained in the optically active or pure form of a single isomer [2]. However, the drugs that are produced by chemical synthesis are usually a mixture of equal parts of two, four or more isomers, depending on the number of asymmetric centers. Accordingly, stereo selectivity in chiral drug bioavailability, distribution, interaction with receptor sites, metabolism and elimination produces differences in isomer activity, ranging from unwanted toxicity to no significance to enhanced activity [3].

There are two fundamental approaches to separating enantiomers: (a) indirect methods of chiral separations which involves the conversion of enantiomers into diastereomeric derivatives by reaction with absolutely optically pure chiral reagents and subsequent separation of the derivatives on commonly used phases; or (b) direct methods involving separation of the racemic drugs to their corresponding enantiomers using chiral stationary phases (CSPs).

3. Stationary phases

Direct methods based on CSPs are preferred since they are rapid and suitable to resolution of racemates on both analytical and preparative scales. Great efforts have been devoted to the development of better methodology for enantioselective chromatography during the past decade, and have resulted in new chiral stationary phases, pioneered by Pirkle [4]. Chiral agents were derivatized and immobilized on the surface of the support (silica gel mostly), and served as the in situ chiral discriminators during the chromatographic process. The preference of chiral stationary phases lies in the inherent advantages of any chromatographic separation, such as the speed of the analysis, the possibility to analyze or purify the enantiomers in complex mixtures, the reproducibility of the analysis and its flexibility. Moreover, analytical chromatographic systems can be adapted to preparative separations, in which pure enantiomers can be collected.

In addition to their distinct practical applicability, chiral stationary phases can uniquely contribute to studies of the nature of molecular recognition. Since the differential retention of enantiomers in the chromatographic system employing chiral stationary phases, can be attributed only to chiral discrimination by the chiral sites, these interactions can be isolated and explored. It has been shown that chromatographic parameters obtained by chiral stationary phases can be sensitive to very subtle differences between the enantiomers. Moreover, chiral stationary phases can be tailor-made to accommodate specific studies of chiral recognition between molecules.

A review by Taylor and Maher [5] describes in detail the principles underlying chiral discrimination by various chiral chromatographic systems, utilizing chiral agents either in the mobile or the stationary phase. Another review by Gubitz [6] describes the application of chiral stationary phases to chiral drugs, emphasizing the main principles of chiral discrimination of the various categories of stationary phases known so far. A conventional classification of types of chiral stationary phases is used here: (A) Chiral affinity by proteins (serum albumin, α 1-acid glycoprotein, ovomucoid and chymotrypsin). (B) Stereoselective access to helical chiral polymers (derivatized or free polysaccharides). (C) Steric interactions between p-Donor p-acceptor type of chiral aromatic amide groups (Pirkle). (D) Host-guest interactions inside chiral cavities (cyclodextrins, crown ethers and imprinted polymers). (E) Ligand exchange (copper ions complexed with chiral moieties).

Most of the analytical methods for pharmaceutical compounds in biological samples use types A-D of the aforementioned stationary phases, and therefore, the discussion will be focused on them. The parameters of importance in chiral recognition by the chromatographic stationary phases will be discussed in each section. It may be generalized that in most cases the difference in steric fit, anchored by hydrogen bonding of the solutes into the chiral environment in the specific discriminating sites, is responsible for the resolution.

The various biological sources from which samples were taken for analysis are specified in the tables, listing compounds of pharmaceutical interest analyzed by the various stationary phases. The purpose of listing the sample source, namely the biological fluid or tissue, is to portray the type of pharmaceutical research that benefits from the availability of the enantioselective analysis. In general, whenever a method describes the enantioselective analysis of drug in plasma, it is being used for therapeutic monitoring of drug levels in the blood, or for studies of enantioselectivity of pharmacokinetic parameters. If the method is applied to plasma and urine, both being analyzed simultaneously, the purpose is probably to track the enantioselective metabolism and/or clearance of drug. If chiral pharmaceutical compounds are analyzed in various tissues, the aim is to study enantioselective absorption and distribution in the various organs (disposition). On many occasions the users develop the analytical procedure to fit their specific needs for stereoselective analysis. The tables can give an indication of the types of problems encountered in the pharmaceutical research that require enantioselective analysis.

4. Development of practical chiral stationary phases

Frequently the methods used for the separations, for monitoring the progress of an asymmetric synthesis or optical purity of the products are chromatographic with liquids, gases, or supercritical fluids as the mobile phase. More recently capillary electrophoresis has been added as an analytical chiral separation method. Over the past three decades, enantioseparations by high-performance liquid chromatography (HPLC) has become increasingly important. It was well known chromatography was one of the most useful methods for the direct resolution of enantiomers. Many workers challenged in this field, but all efforts remained fruitless for many years. The breakthrough was accomplished about 30–40 years ago by the unambiguous separation with some chiral stationary phases by Gil–Av et al. [7] in gas chromatography or by Davankov et al. [8] in liquid chromatography.

However, in those days, efficient chiral stationary phases were very few and not yet commercially available. Therefore, the conversion of enantiomers to their diastereomers with suitable chiral derivatization reagents was usually used for the separation and analysis of chiral compounds. For example, in the case of the separation of eight isomers of allethrin, which contains one chiral centre in the alcohol moiety (allethrolone) and two chiral centers in the acid moiety (chrysanthemic acid), two components obtained by the hydrolysis were converted to their diastereomers and separated to two and four isomers by gas chromatography with conventional achiral stationary phases [9]. Such process was very complicated and the practical effective chiral stationary phases for the direct separation were desired earnestly.

We have prepared many novel chiral stationary phases and examined their enantioselectivities. Fortunately, some of them showed good enantioselectivity, and they could use effectively for the separation and analysis of various chiral compounds. Example of enantioseparation of a drug with stereogenic centers. Enantioseparation of Gantofiban precursors on chiral stationary phases of the poly-(N-acryloyl amino acid derivative)-type studied by M. Schulte et al. [10] and CSP of the poly-[N-(meth-) acryloyl amino acid derivative]-type have been shown to be very useful in the separation of chiral intermediates of drug substances. The high variability of different chiral selectors allows the systematic screening for optimal preparative separation conditions. Due to the high chemical stability of these stationary phases, it is possible to use a wide variety of solvents with different polarities and solvent characteristics.

Within the route towards a final drug compound all chiral intermediates have to be taken into account for a preparative enantioseparation. As it was shown in this study the productivities and in conjunction with this the production costs can vary significantly from one intermediate to another. For the precursors of Gantofiban it turned out that the alcohol intermediate 1 can be separated with the highest productivity, reducing the amount, which has to be carried on during the synthesis at the earliest possible stage.

Nevertheless, the productivity of the optimized separation is low compared to other separations performed in our labs or described in literature. While the best system in this study separates intermediate 1 with a productivity of 150 g enantiomer per day and kg CSP, other separations have been reported with productivities up to 1000 g enantiomer per day and kg CSP [11, 12]. As a rule of thumb, the productivity has to reach at least 400 g enantiomer per d and kg CSP to ensure an economic enantioseparation in production scale. Despite this fact, the separation of enantiomers by means of SMB-chromatography is a very quick and efficient tool to produce the first kg-amounts of developmental drug compounds with high purities and within reasonable time, which is essential to nowadays pharmaceutical research.

Furthermore, as the high molecular diversity of CSP based on polymeric amino acid derivatives offers one of the most flexible approaches for chromatographic enantioseparations, a further increase of the productivity for the Gantofiban precursors can be expected. Four different polysaccharide-type chiral stationary phases (CSP) using polar organic mobile phases such as pure methanol and acetonitrile were considered by Yoshio Okamoto et al. [13] for enantioseparation of the enantiomers of randomly selected chiral drugs and drug analogs of various structural and pharmacological groups. Polysaccharide phenylester type CSP, Chiralcel-OJ although resolving the enantiomers of some chiral drugs was less universal in the combination with methanol and acetonitrile as mobile phases. Among polysaccharide phenylcarbamates amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak-AD) was superior over the corresponding cellulose derivative, cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel-OD). However, another derivative of cellulose, namely, cellulose tris(3,5-dichlorophenylcarbamate) (CDCPC) exhibited higher chiral recognition ability compared to Chiralpak-AD material. They established a fact of applicability of polysaccharide type CSPs in so called polar organic mode as well as shows high potential of CDCPC as a practically useful CSP for High performance liquid chromatography (HPLC) enantioseparations.

In order to facile preparative HPLC enantioseparation of racemic drugs, Immobilized silica gel chiral stationary phases (CSP)s from mono-6^A-azido-6^A-deoxy-perphenylcarbamoylated β -cyclodextrin were prepared by Siu-Choon Ng et al. [14] using an extended application of the Staudinger reaction. Their application in preparative enantioseparations of racemic mixtures was demonstrated using atropine, bendroflumethiazide and four β -adrenergic blocking agents under reversed phase conditions.

L. Chen et al. [15] have been developed successfully a novel chiral stationary phase (PPHCDN7) by immobilization of heptakis(6-azido-6-deoxy-2,3-di-Ophenylcarbamoylated)- β -cyclodextrin (PPHCD) onto the surface of amino-functionalized silica gel via multiple urea linkages derived from an extended application of the Staudinger reaction. A wide range of structurally divergent racemic drugs and other compounds were successfully enantioseparated on this column under both normal and reversed-phase conditions not only by the mechanism of the formation of an inclusion complex, but also by the interaction of hydrogen bonding, π - π interaction and dipole-dipole interaction. β -Adrenergic blockers and racemic tertiary, secondary and primary amines were readily separated using a mixture of methanol and aqueous triethylammonium acetate buffer. The mobile phase constitution, pH value of the system and addition of salts play very important roles in the enantioseparation under the reversed phase. The optimal pH value for the separation falls into the range of 4.65 to 6.30. With atropine and isoproterenol, good enantioseparations with separation factors of $\alpha > 5$ were easily attainable and the stability of the column is excellent and it proved to have good enantioseparation ability under a wide range of conditions.

New high performance chromatographic method on Chirex 3005 column containing amide type chiral stationary phase has been developed by Y. Song et al. [16] for direct separation, also qualitative and quantitative determination of clenbuterol enantiomers. Several parameters such as mobile phase composition, column temperature and flow rate were studied. Baseline enantioseparation was achieved, using the optimized mobile phase of n-hexane-1,2-dichloroethane /methanol (54:38:8, v/v/v) at 178°C and 1.0 ml/min, with the separation factor (α) 1.43 and the resolution factor (R_s) 1.81. The mechanism of separation was also discussed. Standard linear calibration curves were established for the R- and S- enantiomers, over the range of 26.1-1045.8 and 5.7-229.6 nmol/ml, with the correlation coefficient of 0.9999 for both. The limits of detection were 0.47 and 1.04 nmol/ml for R- and S-enantiomers, respectively. Recovery and precision of the method were also evaluated, which had been successfully used to monitor and identify quantitatively the profile of the clenbuterol enantiomers in human serum. This method is simple, fast and reliable.

Enantioselective liquid chromatography on polyacrylamide- and polysaccharide-based chiral stationary phases considered by R. Cirilli et al. [17]. For this purpose optically active synthetic and semisynthetic polymers were utilized as chiral stationary phases (CSPs) for the direct chromatographic enantioseparation of a series of 8-chloro-2,3-dihydro-3-methyl-1,2,5-benzothiadiazepin-4(5H)-one and thione 1,1-dioxide. Evaluation of stereochemical integrity of chiral analytes was assessed by enantioselective temperature and flow-dependent HPLC. A stopped-flow high-performance liquid chromatography (sfHPLC) procedure was developed for the determination of the rate constants and free energy barriers of enantiomerization of enantiomers of 8-chloro-2-(3-methylbut-2-enyl)-2,3-dihydro-3-methyl-1,2,5-benzothiadiazepin-4(5H)-thione 1,1-dioxide (compound 2) in the presence of Chiraspher and Chiralcel OD CSPs. In order to study the chiroptical properties of the individual enantiomers of analytes investigated, semipreparative chromatographic resolutions were performed. The assignment of the absolute configuration was empirically established by comparing the CD spectra of the separated enantiomers with those obtained from structural analogues.

M.G. Schmid et al. [18] investigated enantioseparation of glycyldipeptides by capillary electrochromatography (CEC) on a capillary packed with teicoplanin aglycone immobilized on 3.5 μm silica gel. The results were compared to those obtained with micro-HPLC using the same chiral stationary phase. Polar organic and reversed-phase mode were checked, whereby the latter showed better results. Out of 12 glycyldipeptides investigated, all compounds showed baseline separation with R values up to 20. Plate numbers were in the range of 10 000–300 000/m. The choice of organic modifiers was found to be crucial. In this study they found that addition of methanol increases retention time, whereas increasing amounts of ACN speed up separation. A ternary mixture of ethanol–acetonitrile–aqueous triethylamine acetate solution pH 4.1 was found to be a useful compromise, providing excellent resolution with retention times less than 25 min. Efficiency and resolution were generally found to be higher in CEC than with micro-HPLC.

Following a previous publication, Tao Wang et al. [19] reported additional results on the effects of alcohol mobile phase modifiers on the structure and chiral selectivity of amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD) chiral stationary phase (CSP). Alcohol mobile-phase modifiers of varying size and bulkiness had profound effects on the structure and chiral selectivity of Chiralpak AD. The various alcohol modifiers were all incorporated into the CSP. Chiralpak AD in contact with various alcohol modifiers possessed different structures as evidenced by $^1\text{H}/^{13}\text{C}$ CPMAS solid-state NMR. Compared to linear alcohols (ethanol, 1-propanol and 1-butanol), the branched alcohols (IPA and t-butanol) caused more twisting of the glucose units on the helical structure of the CSP. Other structural changes of the CSP were also observed when various alcohol modifiers of different bulkiness were used. Significant differences in chiral selectivity of the CSP for three pairs of enantiomers were observed when these various alcohol modifiers were used. The differences in chiral selectivity were attributed to CSPs observed structural changes caused by the alcohol modifiers of different bulkiness. It is believed that the combined effect of the various structural changes creates a specific stereo

environment on the chiral cavities, which control the chiral selectivity through steric fit of the solute into the cavities along with CSP-solute interactions such as hydrogen bonding, dipole-dipole and π - π interactions. Therefore, the bulkiness of the alcohol modifier plays an important role in determining the chiral selectivity of the CSP. Finally, the change of concentration of an alcohol modifier (such as *t*-butanol) in the mobile phase can cause changes in structure and chiral selectivity of the Chiralpak AD.

X. Zhang et al. [20] have developed direct liquid chromatographic separations of the enantiomers of metoprolol and bisoprolol, using (R)-1-naphthylglycine and 3,5-dinitrobenzoic acid as chiral stationary phase (CSP). The proposed stationary phase is an efficient CSP to separate some β -blockers. The separations were achieved in a normal phase system employing a mobile phase containing *n*-hexane, 1,2-dichloroethane and methanol. Column efficiency was strongly dependent on the composition of the mobile phase. Metoprolol and bisoprolol can be separated into their both enantiomers, achieving baseline separation under mild separational conditions. Polarity of the mobile phase greatly affects the separation. The best mobile phase for metoprolol enantiomer separation is V (hexane):V (1,2-dichloroethane):V (methanol) 65:25:10, and for the bisoprolol enantiomers, V (hexane):V (1,2-dichloroethane):V (methanol) 60:30:10. Column temperature and flow-rate also produce some effects upon separation. The optimum temperature for enantiomer separation is 15.0 °C and the optimum flow-rate is 0.600 ml/min. Under the optimal conditions, linear responses for (R)-metoprolol and (S)-metoprolol are obtained in the range of 0.079-1.38 and 0.015-5.80 mg/ml, with detection limits of 0.008 and 0.002 mg/ml, respectively. As for bisoprolol, the linear ranges of (R)-isomer and (S)-isomer are 0.05-1.31 and 0.02-1.00 mg/ml with detection limits of 0.001 and 0.008 mg/ml, respectively. The relative standard deviation (R.S.D.) of each enantiomer did not exceed 0.90%. The procedure suggested in the present method is quite simple since no analyte derivatization is required, the mobile phase used being most common and method repeatability is good. This assay provides a convenient method for further investigation of enantiomeric separation and detection of other β -blocking pharmaceuticals.

To improve the therapeutic potential of anti-HIV nucleoside analogues (d4T, AZT, 3TC and ddI), the delivery of the corresponding monophosphate from neutral, membrane-permeable prodrugs has been realised by the synthesis of lipophilic phosphoramidate triester prodrugs, such as the simple phenyl-L-alaninephosphate derivatives. However, the present non-stereoselective synthesis results in a mixture of 1:1 diastereomers, which differ from the configuration of the phosphorus atom asymmetric center. Since each diastereomer may have different biological activity and pharmacokinetic profile, analytical methods have to be developed for their separation. For this aim N. Mesplet et al. [21] demonstrated the ability of a polysaccharide-type chiral stationary phase (Chiralcel OD-RH) to resolve such diastereomers in reversed-phase high performance liquid chromatography and found that it has been a very useful material to carry out HPLC separation of phosphoramidate diastereomers. The thermodynamic study has shown that the solute transfer is enthalpically driven; however, increasing temperature was detrimental to the resolution. Quantitative analysis has shown good results in terms of linearity, accuracy and repeatability, in spite of passable LOD and LOQ values. Nonetheless, the viability of HPLC - ESI-MS coupling enables to enhance the detection sensitivity and provides a unique selectivity. Further HPLC -ESI-MS -MS experiments would enable to reach greater sensitivity limits. Finally, the next step of this work would be the isolation of each pure diastereomer (preparative scale) as this would be essential for HPLC identification and further pharmacological studies.

Mono(6^A-N-allylamino-6^A-deoxy)perphenylcarbamoylated β -cyclodextrin is a novel cyclodextrin derivative that was synthesized and applied as a chiral stationary phase for HPLC by Xianghua Lai and Siu-Choon Ng [22]. Mono-6-deoxy-6-(*p*-tolylsulfonyl)- β -cyclodextrin 1 was readily converted to the key intermediate 2 in high purity and good yield by refluxing in allylamine for 5 h and then precipitating the product in acetonitrile [23]. Reaction of 2 with phenyl isocyanate afforded 3. Thereafter, hydrosilylation of 3 with (EtO)₃SiH in the

presence of a catalytic amount of tetrakis(triphenylphosphine) platinum(0) gave the reactive siloxane 4, which was directly immobilized onto the surface of silica gel to give the CSP 5 and, structurally well-defined chiral stationary phase capable of enantioseparation of a variety of racemic drugs.

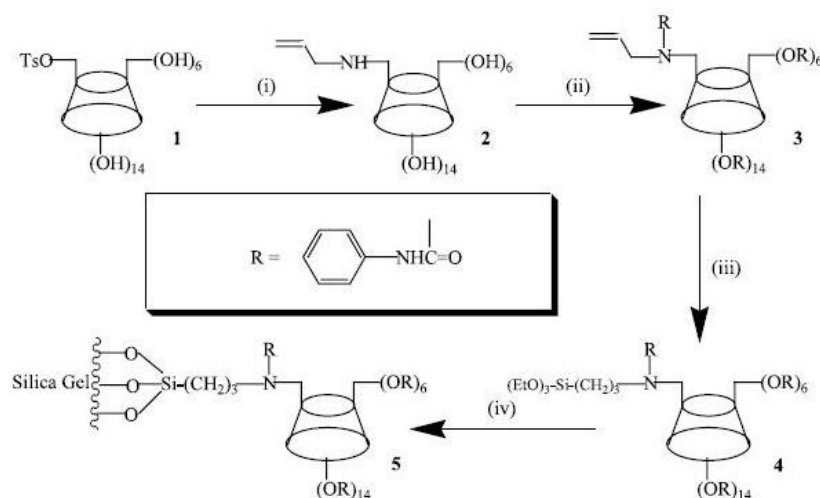


Fig. 1. Synthetic route to CSP 5. Reagents and conditions: (i) $\text{CH}_2=\text{CHCH}_2\text{NH}_2/\Delta$; (ii) $\text{PhN}=\text{C}=\text{O}/\text{C}_5\text{H}_5\text{N}/\Delta$; (iii) $(\text{EtO})_3\text{SiH}/\text{cat. Pt}(\text{PPh}_3)_4/\text{THF}/\Delta$; (iv) silica gel/ $\text{CH}_3\text{C}_6\text{H}_5/\Delta$.

It has been demonstrated that three types of CSPs based on cellulose phenylcarbamate, cellulose 4-methylbenzoate and cellulose 3,5-dimethylphenyl-carbamate were successfully prepared with the bifunctional reagent of 3-(triethoxysilyl)propyl isocyanate (TEPI) that was initially adopted as a spacer reagent to prepare the bonded types of chiral stationary phases (CSPs) with cellulose derivatives [24], for effective enantioseparations. The silica-based CSPs were chemically prepared with non-regioselective and regioselective approaches and their chiral resolving capabilities were evaluated in terms of HPLC resolution of test enantiomers. It was observed that the enantioselectivities on the non-regioselectively prepared CSPs increased with the increment of the spacer reagent used for the synthesis. The regioselectively prepared CSP generally showed a higher resolution power than the non-regioselectively prepared CSP for the test enantiomers, while the latter phase showed great advantage from the view of rapid preparation. In addition, chiral recognition of the prepared CSPs was affected by the properties of the used silica matrices. The CSPs based on the silica gel with pore size of 300 Å exhibited higher enantioselectivities than those prepared with pore size of 200 Å.

M.M. Hefnawy and H.Y. Aboul-Enein [25] developed and validated an enantioseparation high performance liquid chromatographic (HPLC) method to determine d-(+)- and l-(-)-vesamicol in human plasma. The vesamicol enantiomers were separated with a vancomycin chiral stationary phase. The assay involved the use of an efficient solid phase extraction procedure for plasma sample clean up prior to HPLC analysis utilizing a C18 Bond-Elute column. Chromatographic resolution of the vesamicol enantiomers was performed on a vancomycin macrocyclic antibiotic chiral stationary phase (CSP) known as Chirobiotic V with a polar ionic mobile phase (PIM) consisting of methanol:glacial acetic acid:triethylamine (100:0.1:0.05 (v/v/v)) at a flow rate of 1.0 ml/min and UV detection set at 262 nm. All analyses were conducted at ambient temperature. The method was validated over the range of 1–20 µg/ml for each enantiomer concentration ($R_2 > 0.999$). Recoveries for d-(+)- and l-(-)-vesamicol enantiomers were in the ranges of 96–105% at 3–16 µg/ml level. The total run time for this method is 15 min, which allows processing of over 90 samples per day. This method has provided good sensitivity and excellent precision and reproducibility.

Tamsulosin hydrochloride is used in the treatment of benign prostatic hypertrophy and was approved by the FDA in May 1997 after a review lasting 12 months [26]. The drug is a new antagonist of α_1A adrenoceptors, which causes smooth muscle relaxation. For the chiral separation of an antagonist of α_1A adrenoceptors, tamsulosin and its S-isomer a high-performance liquid chromatographic (HPLC) method using cellulose Tris (3,5-dimethylphenylcarbamate) as a chiral stationary phase was developed by Z. Zhang et al. [27]. Baseline separation of the isomers was achieved within 35 min on a CHIRALCEL OD-RH column with a binary solvent mixture of 50 mmol l^{-1} KPF6–acetonitrile (v/v (70:30), pH 5.0) as the optimized mobile phase. The detection limits and quantification limits of both R-isomer and S-isomer were 0.11 and 0.44 ng, respectively. The R.S.D. values of peak-area for the two isomer were 0.42% (of peak-height: 0.77%) for R-isomer and 0.64% (of peak-height:0.92%) for S-isomer ($n = 5$). From the above results, it can be seen that the proposed method is simple, sensitive and accurate, and can be used for the quality evaluation of tamsulosin hydrochloride.

Comparison of different eluent compositions on enantioseparation showed that even small changes in the composition may affect the resolution significantly. K. Pihlainen and R. Kostianen studied Enantioseparation of nine amphetamine derivatives, methorphan and propoxyphene by comparing two different chiral stationary phases, macrocyclic antibiotic vancomycin and native β -cyclodextrin (β -CD) [28]. Effects of 46 eluent compositions on enantioseparation in reversed-phase (RP) and polar organic phase modes were investigated. β -CD was found to be more suitable for chiral analysis of amphetamine derivatives than the vancomycin column. However, the vancomycin column, using a polar organic phase was more suitable for enantioseparation of methorphan and propoxyphene than the β -CD column. The β -CD column allowed not only separation of enantiomers of the amphetamine derivatives, but also separation of the amphetamines from each others using UV detection. The high concentrations of non-volatile buffers caused severe signal suppression in ESI–MS. However, it was shown that non-volatile TEAA can be replaced with ammonium acetate without decreasing resolution. The repeatability and sensitivity of the LC–ESI–MS/MS method were shown to be acceptable and its applicability to authentic sample material was assessed.

Quinine carbamate-type weak chiral anion-exchange selectors (SOs) and the respective chiral stationary phases (CSPs) have been used for the direct liquid chromatographic enantiomer separation of a wide range of chiral acids. K. Gyimesi-Forrás et al. [29] investigated Practical and mechanistic aspects of the retention and chiral discriminating ability of quinine carbamate type CSPs for a set of neutral polar imidazo-quinazoline-diones as potential NMDA and/or AMPA receptor antagonists. They demonstrate that these CSPs can also be extended to chiral discrimination of a set of neutral polar potential NMDA (N-methyl-d-aspartic acid) and/or AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) antagonist imidazo-quinazoline-dione derivatives (selectands, SAs) using acetonitrile and methanol containing hydro-organic and buffered mobile phases. The influence of ionic strength of the mobile phase, type and concentration of organic additive, structural variation of analytes and column temperature on the overall enantiomer separations were systematically investigated to gain insight into the overall chiral recognition mechanism. The tert-butyl carbamoyl quinine SO proved to be suitable for the direct enantiomer separation of neutral analytes under the hydro-organic conditions.

Varying the structure of the SO, the chiral recognition was influenced significantly, indicating the importance of steric requirements for the binding sites. It was shown that the buffer concentration does not play a major role in the retention; however it has reasonable influence on overall enantioselectivity and efficiency. Acetonitrile has a stronger effect on elution than methanol; this supports an enhanced chiral discrimination process for most of the investigated analytes. Apart from Van der Waals interactions and hydrogen bond formations, π – π interactions seem to play a crucial role in the enantiomer separation. Regarding the strong effect of the type and position of substituents on the phenyl ring, steric hindrance phenomena and conformational aspects seem to be responsible for the lack of enantiomer separation obtained

for analyte 6. These results were also demonstrated by molecular modeling. Thermodynamic analysis showed that enantiomer separation was predominantly enthalpically driven for the selected analytes at the temperature employed.

Using molecular modeling, molecular dynamics, we can study the chiral discrimination leading to the resolution of organophosphonate derivatives enantiomers on pirkle type chiral stationary phase, by calculating the energy of the complex of CSP and enantiomers. For this purpose molecular modeling and molecular dynamics (MD) have been used by G.-S. Yang et al. [30] to study the chiral discrimination and interaction energy of organophosphonate in N-(3,5-dinitrobenzoyl)-S-leucine chiral stationary phase (CSP). The elution order of the enantiomers can be predicted from the interaction energy. Quantitative structure-retention relationship (QSRR) has also been used as an alternative method to confirm the elution order of enantiomers. Molecular mechanics, molecular dynamics and QSRR can be useful methods to the study of chiral discrimination.

A novel chiral stationary phase with amino-linked and immobilized mono(6^A-N-allylamino-6^A-deoxy)-perphenyl carbamoylated β -cyclodextrin onto the surface of silica gel via hydrosilylation has been conveniently prepared by Xiang-Hua Lai and Siu-Choon Ng [31]. The synthesis of amido-bonded perphenylcarbamoylated β -cyclodextrin (CSP 5) was affected in a relatively straightforward manner according to the synthetic route depicted in Fig. 2. Mono[6^A-(p-toluenesulfonyl)-6^A-deoxy]- β -cyclodextrin, compound 1 [32, 33] was readily converted to the key intermediate 2 in high purity and good yield by refluxing in allylamine for 5 h and then precipitating the product in acetonitrile [34]. Reaction of 2 with phenyl isocyanate afforded 3. Thereafter, hydrosilylation of 3 with (EtO)₃SiH in the presence of catalytic amount of tetrakis(triphenylphosphine)platinum(0) gave the reactive siloxane 4, which was directly immobilized onto the surface of silica gel to afford the CSP 5. Then the chromatographic properties of this column were tested with a wide range of structurally diverse racemic compounds and drugs under reverse phases. Also Separation mechanisms involved are also discussed. The enantioseparation data indicate that the current synthetic procedure can afford improved CSPs.

Microbore column packed with streptavidin particles was used by C. Ravelet et al. [35], at various temperatures (0–24°C), to separate the adenosine enantiomers by HPLC. Using an aqueous mobile phase, the apparent enantioseparation was high for a small molecule, varying from 11.5 at 0°C to 6.2 at 24°C. From the experiments carried out with a streptavidin–biotin complex stationary phase, it was demonstrated that the blockage of the biotin sites of the immobilized streptavidin was responsible for a strong decrease in the enantioselectivity via a direct and/or an indirect effect. From the analysis of the concentration dependencies of the solute retention factor, it was also shown that a reduction of the d-adenosine specific binding sites occurred at the lowest temperature. The thermodynamic parameters determined from the van't Hoff plots indicated that the d-adenosine binding to the streptavidin specific sites was enthalpically driven. The present results demonstrate that streptavidin is a useful chiral stationary phase for the separation of the adenosine enantiomers. In addition, the study on the column temperature influence on the retention and chiral recognition shows that the d-adenosine saturation capacity of the streptavidin column decreases at 0°C. Finally, the solute binding to the streptavidin specific sites is enthalpically governed.

The bonded-type of CSPs with cellulose phenylcarbamate derivatives having methacrylate groups were efficiently synthesized with regioselective and non-regioselective procedures [36]. These derivatives were chemically immobilized onto a vinylized silica gel, respectively, via a radical co-polymerization reaction. The immobilization was efficiently attained using a small amount of AIBN. The chiral recognition abilities of the prepared chiral stationary phases (CSPs) were evaluated by HPLC resolution of test enantiomers. It was observed that most of the enantiomers were completely resolved with markedly high column efficiency of 30,000–40,000 plates per metre for the eluted peaks. The effect of the amount of methacryloyl chloride used for preparation on resolution was investigated. A direct comparison of the chiral recognition ability

was made on the regioselectively and non-regioselectively prepared CSPs. In addition, the chemically bonded-type of CSPs were found to be relatively stable with addition of solvents such as tetrahydrofuran (THF) and chloroform into the mobile phase, which can lead to the dissolution of cellulose derivatives on the coated CSPs. Thus the choice of solvents used as the mobile phase is greatly extended and better resolution of several test enantiomers was observed on the prepared CSPs with THF and chloroform as a composition in the mobile phase which makes it possible to extend the application range on the newly prepared CSPs for separation of enantiomers.

Novel chromatographic method to rapidly and simply characterize the pharmacokinetics of benidipine enantiomers in human plasma was developed by W. Kang et al. [37]. The stereoisomers of benidipine were extracted from plasma using diethylether under alkaline conditions. After evaporating the organic layer, the residue was reconstituted in the mobile phase (methanol:acetic acid:triethylamine, 100:0.01:0.0001, v/v/v). The enantiomers in the extract were separated on a macrocyclic antibiotic (Vancomycin) chiral stationary phase column. The mobile phase was eluted at 1 ml/min and was split by an interface. One-fifth of the eluent was used to quantify both isomers in a tandem mass spectrometer in multiple reaction monitoring mode. The coefficient of variation of the precision of the assay was less than 8%, the assay accuracy was between 93.4 and 113.3%, and the limit of detection was 0.05 ng/ml for 1ml of plasma. The method described above was used to measure the concentration of both benidipine enantiomers in plasma from healthy subjects who received a single oral dose of a racemate of 8mg benidipine. The C_{max} and AUC_{inf} values of (+)- α -benidipine were higher than those of (-)- α -benidipine by 1.96- and 1.85-fold, respectively ($p < 0.001$), whereas, the T_{max} and $t_{1/2}$ for each of the benidipine stereoisomers were not significantly different. In conclusion this method is suitable for clinical pharmacokinetic studies of benidipine enantiomers after the oral administration of a benidipine racemate.

Z.D. Zhai et al. [38] developed Novel HPLC methods which was simple, rapid, economic, sensitive and robust analytical for the analytical and semipreparative enantioseparation of new antidepressant drug mirtazapine enantiomers. At analytical scale, the separation of the mirtazapine enantiomers was investigated using both cellulose and amylose tris(3,5-dimethylphenylcarbamate) (CDMPC and ADMPC) chiral stationary phases under normal-phases and polar organic modes. Good baseline enantioseparation was achieved using cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phases under both normal-phases and polar organic modes. Furthermore, the elution order of mirtazapine enantiomeric pairs was reversed by changing the stationary phase from the amylose-based ADMPC-CSPs to its cellulose-based counterpart, CDMPC-CSPs. The validation of the analytical methods including linearity, limit of detection (LOD), limit of quantification (LOQ), recovery and precision, together with the semipreparative resolution of mirtazapine racemate were carried out using cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phases and methanol as mobile phase without any basic additives under polar organic mode. At analytical scale, the elution times of both enantiomers were less than 6 min at normal temperature and 1.0 ml/min, with the separation factor (α) 1.99 and the resolution factor (R_s) 3.56. Then, the analytical methods were scaled up to semipreparative loading to obtain small quantities of both mirtazapine enantiomers. At semipreparative scale, about 16 mg/h enantiomers could be isolated and elution times of both enantiomers were less than 10 min at 2.0 ml/min. To increase the throughput, the technique of boxcar injections was used. One enantiomer ((-)-(R)-mirtazapine) was isolated with purity of >99.9% e.e. and >98.0% yield and another ((+)-(S)-mirtazapine) was isolated with purity of >97.0% e.e. and >99.0% yield. In addition, optical rotation and circular dichroism (CD) spectroscopy of both mirtazapine enantiomers isolated were also investigated. The analytical method was successfully scaled up to semipreparative loadings. Small quantities of high purity of both mirtazapine enantiomers were obtained in short time. With the use of preparative chiral column (i.d. >2 cm) and automated collection equipment, large amount of enantiopure mirtazapine could be easily and quickly acquired on the base of the method developed in this

paper. In addition, optical characters of individual enantiomers prepared were studied by optical rotation and CD spectroscopy. Single mirtazapine enantiomers were found having comparatively larger absolute value of optical rotation and quite stable in configuration in 1 month.

For enantioselective analysis of synephrine in natural products, F. Pellati et al. [39] developed an enantioselective LC method with photodiode array detection (PAD) for the enantioseparation of (\pm)-synephrine from *C. aurantium* L. var. *amara* fruits and phytotherapeutic derivatives by using a protein-based chiral stationary phase with cellobiohydrolase as the chiral selector (Chiral-CBH). Analyses were carried out on a Chiral-CBH column (100 \times 4.0mm i.d., 5 μ m), with a mobile phase consisting of 2-propanol (5%, w/w) in sodium phosphate buffer (pH 6.0; 10 mM) and disodium EDTA (50 μ M). The flow rate was 0.8 mL/min. Detection was set at 225 nm. To identify the order of elution, the racemate was resolved by the preparation of suitable diastereoisomeric salts with antipodes of appropriate organic acids. Isolation of synephrine from *C. aurantium* fruits and phytoproducts was performed by solid-phase extraction (SPE) with a strong cation exchange phase.

The method developed was validated and was found to be linear in the 0.40–40.14 μ g/mL range ($r^2 = 1.000$, $P < 0.0001$) for both synephrine enantiomers. The limit of detection (LOD) for each enantiomer was 0.04 μ g/mL. The limit of quantification (LOQ) for each enantiomer was 0.13 μ g/mL. Intra-day precision (calculated as %R.S.D.) ranged from 0.03 to 0.24% for (–)-synephrine and from 0.03 to 0.35% for (+)-synephrine. Inter-day precision (calculated as %R.S.D.) ranged from 0.07 to 1.45% for (–)-synephrine and from 0.06 to 1.26% for (+)-synephrine. Intra- and inter-day accuracies (calculated as %recovery) were in the ranges of 97.4–100.6 and 98.0–101.6% for (–)-synephrine, and in the ranges 97.0–101.5 and 98.1–102.8% for (+)-synephrine. The results of the application of the method to the analysis of *C. aurantium* samples showed that (–)-synephrine was the main component. (+)-Synephrine was not detected in *C. aurantium* fruits and was present in low concentration in the phytoproducts.

The validation procedure proved that the method has good linearity, accuracy, precision and sensitivity. The practical applicability of this procedure was tested by assaying synephrine enantiomers in *C. aurantium* fruits and phytoproducts. It was found that strong cation-exchange SPE is suitable for sample clean-up before LC analysis. The present method enabled us to measure synephrine enantiomers in *C. aurantium* samples and could be useful for further investigations on the possible racemization of (–)-synephrine during the extraction of *C. aurantium* fruits. This assay provides a convenient method for the future investigation of the enantiomeric separation and detection of synephrine enantiomers in various vegetable matrices and biological fluids.

Sample solubility in the mobile phase and enantioselectivity are key factors in chiral preparative chromatography. In the search for a high throughput process for production of pure enantiomers, the rational design of the mobile phase and the selection of a suitable chiral stationary phase (CSP) are essential. However, one may sometimes be faced with the incompatibility between the CSP and the preferential eluent for sample solubility. Such a limitation may be circumvented by using an immobilized CSP such as CHIRALPAK[®] IA. T. Zhang et al. [40] worked on Optimization of the chiral separation of a Ca-sensitizing drug (EMD 53986) on an immobilized polysaccharide-based chiral stationary phase on CHIRALPAK[®] IA in terms of sample solubility, enantioselectivity and preparative productivity. The approaches for method optimization and the impact of sample solubility on productivity were discussed. The preparative potential of CHIRALPAK[®] IA was also demonstrated. Although this piece of work deals with the optimization of a specific chiral separation for a given compound, some general conclusions can be derived. Sample solubility in mobile phase has a direct impact on productivity in preparative separation of enantiomers by chromatography. In practice, the solvents having high dissolution power for the solute should be considered as mobile phases or mobile phase components. From the method development viewpoint, a compromise of sample solubility, enantioselectivity, resolution and retention time is often needed. Owing to its solvent versatility and high chromatographic performance, CHIRALPAK[®] IA allows method

development directly guided by sample solubility with the possibility to improve the enantioselectivity. This CSP has proven itself to be versatile for both analytical and preparative applications.

Enantiomeric separation of basic and acidic compounds of pharmaceutical interest was achieved by using nano-liquid chromatography utilizing laboratory-assembled instrumentation. Experiments were carried out in a fused silica capillary of 75 μm I.D. packed by us with modified silica stationary (5 μm particles), the detection, was done on-line at 195 nm. G. D'Orazio et al. [41] studied enantiomeric resolution of alprenolol, atenolol, metoprolol, oxprenolol, pindolol, propranolol (basic compounds) and some acidic analytes, namely 2-[(5'-benzoyl-2'-hydroxy)phenyl]propionic acid (DF1738Y), 2-[(4'-benzoyloxy-2'-hydroxy)phenyl]propionic acid (DF1770Y), ketoprofen, indoprofen and suprofen by nano-LC utilizing mobile phases containing methanol-acetonitrile-ammonium formate or acetate. The effect of mobile phase composition (buffer type and concentration, organic modifier type and concentration) on chiral resolution (R_s), retention factor (k) and retention time (t_R) was also investigated. The vancomycin chiral stationary phase exhibited good enantio-recognition capability towards all studied compounds by selecting the appropriate mobile phase taking into account the pH of the buffer, the type and concentration of the organic modifier. Acidic compounds were better resolved at lower pH than basic one.

The nano-LC method was validated using R-(+)-propranolol as an internal standard finding good repeatability of retention time, detection limit, correlation coefficient and recovery and applied to the assay of a pharmaceutical formulation containing a racemic mixture of metoprolol present in a pharmaceutical formulation; the method was successfully applied to the assay of metoprolol enantiomers compared to other related analytical methods, e.g., HPLC, nano-LC allows achieving good results at lower costs and minor environmental impact due to the minute amount of chiral stationary phases and lower volumes of mobile phases, respectively. The limitation of this nano-technique is the low injected volume samples that may represent a disadvantage due to the sensitivity necessary for practical applications, e.g., in biological fluids, however this problem can be resolved using different sampling approaches of sample pre-treatment. In order to apply the nano-LC method to practical analysis, they were carried out in our laboratory studies dealing with focusing effect or sample pre-treatment (liquid-liquid extraction).

An improved and validated HPLC method for Separation and determination of bicalutamide enantiomers in drugs and pharmaceuticals on two different polysaccharide-based chiral stationary phases, viz., Chiralcel OD-H (cellulose tris-(3,5-dimethylphenylcarbamate)) and Chiralpak AD-H (amylose tris-(3,5-dimethylphenylcarbamate)) was developed by R. Nageswara Rao et al. [42]. The chiral recognition mechanism on these CSPs is generally due to the formation of solute-CSP complexes through inclusion of the enantiomers in to the chiral cavities in the higher order structures of the CSPs [43, 44]. In case of CSPs with carbamate derivatives, the binding of the solutes to the CSPs is through interactions between the solutes and the polar carbamate groups on the CSPs [45, 46]. The carbamate groups can interact with solutes through hydrogen bonding using C=O and NH groups, and through dipole-dipole interaction using C=O moiety. In the present study the available functional groups on the solutes are OH, and NH which can form hydrogen bonds with the C=O group on the CSPs. Wainer et al.[45] **Error! Bookmark not defined.** have reported that the solutes having aromatic functionalities could provide additional stabilizing effect to the solute-CSP complex by insertion of the aromatic ring into the chiral cavity. In the present case, this type of stabilization effect may be possible due to the presence of the aromatic functionality on the solutes. Chiralcel OD-H column did not show any selectivity for the BCT enantiomers with 2-propanol, while it showed some selectivity with ethanol. 10% of ethanol on this column

Whereas Chiralpak AD-H column has shown excellent selectivity for the BCT enantiomers with decreased retention. These differences in chiral recognition mechanism could be attributed to the different configurations of the glucose residues (β and α linkages) and higher order

structures of chiral stationary phases of OD-H and AD-H columns [44]. Finally, Chiralpak AD-H was chosen for further development. Chiralpak AD-H (250mm×4.6 mm; particle size 5µm) column using n-hexane:2-propanol (65:35 v/v) as mobile phase at a flow rate of 1.0 ml/min at 25°C has shown excellent selectivity for BCT enantiomers. Baseline separation with $R_s \geq 6.0$ was achieved between the two enantiomers within 10 min on Chiralpak AD-H. The detection was made at 270 nm using UV detector while a polarimetric detector connected in series was used for identification of enantiomers. Also the effect of temperature and organic modifiers such as 2-propanol, ethanol on enantioselectivity and resolution of enantiomers were evaluated to optimize the mobile phase composition. The enantiomeric separation was found to be an enthalpy driven process. The method was validated with respect to accuracy, precision, linearity, LOD, LOQ and robustness. The developed method is quite simple, rapid, sensitive and enantioselective and could be of use for determination of enantiomeric purity of BCT in bulk drugs and pharmaceuticals. Improvement in terms of rapidity, resolution, sensitivity and ruggedness compared to that of Torok et al. [47] was achieved on Chiralpak AD with 35% of 2-propanol as an organic modifier. Further, it is suitable for isolation and purification of individual enantiomers by semi preparative HPLC.

A comparison was performed on both new immobilized and conventional coated amylose tris-(3,5-dimethylphenylcarbamate) chiral stationary phases (Chiralpak IA and Chiralpak AD, respectively) by Hassan Y. Aboul-Enein et al. [48] using methanol as mobile phase and ethanolamine as an organic modifier (100:0.1, v/v) for the enantioselective analysis of some β -blockers in LC. The separation factor and retention time of the enantiomers on both columns under the same conditions were different albeit the similarity in structure of the stationary phases in both columns. The separation, retention and elution order of the enantiomers on both columns under the same conditions were compared. The effect of the immobilization of the amylose tris-(3,5-dimethylphenylcarbamate) chiral stationary phase on silica (Chiralpak IA) on the chiral recognition ability was noted when compared to the coated phase (Chiralpak AD) which possesses a higher resolving power than the immobilized one (Chiralpak IA). A few racemates, which were not or poorly resolved on the immobilized Chiralpak IA were most efficiently resolved on the coated Chiralpak AD. However, the immobilized phase withstands solvents like dichloromethane when used as an eluent or as a dissolving agent for the analyte. The versatility of the immobilized Chiralpak IA in monitoring reactions performed in dichloromethane using direct analysis techniques without further purification, workup or removal of dichloromethane was studied on a representative example consisting of the lipase-catalyzed irreversible transesterification of a β -blocker using either vinylacetate or isopropenyl acetate as acyl donor in dichloromethane as organic solvent.

The immobilization of the amylose tris-(3,5-dimethylphenylcarbamate) on silica did affect the chiral recognition ability showing a lower resolving ability than the coated Chiralpak AD. However, the versatility of the Chiralpak IA in monitoring reaction performed in prohibited LC solvents like DCM is demonstrated in a selected example consisting of the lipase-catalyzed kinetic resolution of propranolol in DCM as organic solvent which reflect the useful application of this newly developed phase in chiral analysis.

Simulated moving bed (SMB) is a large-scale version of traditional high-performance liquid chromatography (HPLC), but unlike normal HPLC, SMB operates continuously, without loss of the enantiomeric purity in outlet streams. This process consists of simulating the counter current movement of the adsorbent bed by switching the positions of inlet and outlet streams to produce two outlet streams, one of which is rich in the more adsorbed component (extract stream), while the other is rich in the less adsorbed component (raffinate stream). This procedure is appropriate for binary separations such as required for racemates. The SMB system has been used to separate components from racemic mixtures [49], since it can provide two enantiomers of a chiral molecule with sufficiently high purity and quantities for clinical tests or even production stages. The variety of chiral selectors used as stationary phase and the vast number of racemic mixtures produced by the pharmaceutical industry make this technique a powerful tool and provide a

stimulating and challenging area of research for both laboratory scale studies and production plant designs. Also cellulose tris(3,5-dimethylphenylcarbamate) is frequently used as a stationary phase for enantiomeric separations because of its attractive properties, including high enantioselectivity, high loading capacity and good mechanical stability.

C.C. Santana et al. [50] investigated the usefulness of cellulose tris (3,5-dimethylphenylcarbamate) as the stationary phase in eight columns in an LMS system and of ethanol and hexane mixtures as the mobile phases for the chromatographic separation of potential pharmaceutical intermediates. Using adsorption equilibrium data, they determined the optimal operational conditions for the separation of the N-Boc-4-[p-chloro-phenyl]-2-pyrrolidone enantiomers – a baclofen precursor – in a semi-preparative scale simulated moving bed unit. This unit was used to obtain high purity enantiomers on a scale of 1 g/day.

The laboratory-scale SMB unit consisted of eight stainless steel columns (0.8 cm×15 cm) distributed in four pairs of two columns each (Fig. 2). The desorbent was recycled outside the series of columns by using a multiposition valve instead of a solvent reflux pump. Other four multiposition valves changed the positions of the feed, desorbent inlet, raffinate and extract outlets at preset switch times. These valves were connected to four semi-preparative liquid chromatographic pumps (Shimadzu LC-6AD). The multiposition valves (Valco Instruments Co.) were electrically controlled and linked to a computer by a data-acquisition board. Each valve operated the unit automatically changing positions at pre-determined time intervals. These valves were controlled by a program developed with Labview[®] software.

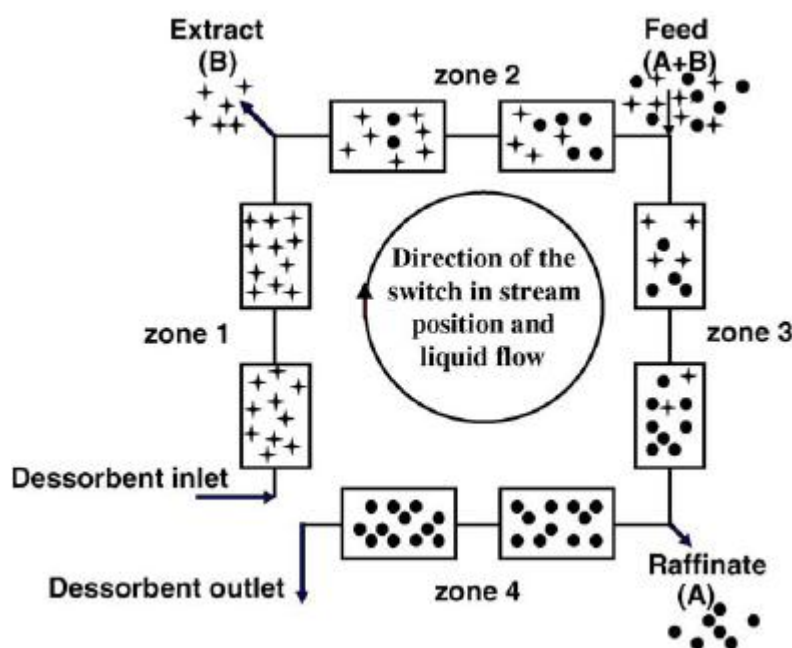


Fig. 2. Basic scheme of the SMB laboratory-scale unit.

The outlet streams were analyzed by an on-line system that consisted of a UV–vis spectrophotometric unit, a polarimeter, and HPLC. Enantiomeric purities of up to 97% were obtained for the raffinate stream and up to 90% for the extract stream. The whole path from synthesis to final product was covered in this work (synthesis of racemate, synthesis and packing of the stationary phase, design and realization of the separation). The application of equilibrium theory based on linear isotherms was useful for determining the operating conditions needed to provide a good separation within the range of feed concentrations used herein. The unit had a processing capacity of 7.2 g of racemic mixture/[day×kg of adsorbent].

A normal-phase isocratic chiral liquid chromatographic method has been developed and validated by J.A. Sellers et al. [51] for atomoxetine hydrochloride. Method development

strategies included (a) evaluation of polysaccharide-based chiral stationary phases with nonaqueous mobile phases, (b) the use of an octyl stationary phase with a sulfated- β -cyclodextrin mobile phase additive, and (c) capillary electrophoresis using a single isomer heptakis-6-sulfato- β -cyclodextrin modifier. All three approaches yielded acceptable conditions for the separation of atomoxetine from related molecules with the former fully validated and the latter two held as alternatives if needed. A polysaccharide-derived CSP (Chiralcel OD-H) with mobile phase of hexane/IPA/DEA/TFA (85/15/0.15/0.2, v/v/v/v) was found to be successful in separating atomoxetine hydrochloride from the undesired S-enantiomer, meta and para-positional isomers and des-methyl related impurity. Preliminary conditions based on the cellulose CSP were found to be insufficiently robust for long-term control use and were optimized further. It was noted that in addition to a DEA additive in IPA and hexane to improve peak shape and efficiency, the addition of TFA to the mobile phase was necessary to facilitate method robustness.

Furthermore, a wider set of parameters were also investigated and optimized including the stationary phase itself and mobile phase solvent composition. It was concluded for atomoxetine, as others have previously noted for other compounds, that selectivity, chromatographic performance and enantiomeric peak elution could be influenced and controlled by switching from an amylose to a cellulose based polysaccharide phase or through the use of either a linear or branched alcohol mobile phase modifier. During the method development, additional chromatographic and electrophoretic separation conditions were also considered. It was shown that selectivity for atomoxetine and related molecules could also be attained using either a chiral mobile phase additive with a C8 column or with CE when a derivatized cyclodextrin was added to the background electrolyte. The final LC conditions were fully validated according to the ICH guidelines with acceptable results obtained for specificity, linearity, accuracy, repeatability, intermediate precision and quantitation limit.

High-performance liquid chromatographic methods for separation of racemic doxazosin mesylate and its synthetic precursors on polysaccharide based stationary phases viz., amylose tris-(3,5-dimethylphenylcarbamate) (Chiralpak AD-H) and cellulose tris-(3,5-dimethylphenylcarbamate) (Chiralcel OD-H) were developed by R. Nageswara Rao et al. [52]. The base line separation with $R_s > 1.50$ was obtained using a mobile phase containing n-hexane-alcohol-0.1% diethylamine (ethanol, 1-propanol and 2-propanol) in various proportions. Chiralpak AD-H column containing tris-(3,5 dimethylphenylcarbamate) as a stationary phase was found to be well adopted for the determination of I, II and DXZN when compared to Chiralcel OD-H. The effects of organic modifiers viz.; ethanol, 1-propanol, 2-propanol and temperature on selectivity and resolution was studied. The optimum separation was obtained on Chiralpak AD-H column with n-hexane:ethanol:2-propanol:diethylamine (70:23:7:0.1, v/v/v/v) as mobile phase and UV-detector at 240 nm. The separation was found to be an enthalpy driven process and the method was validated with respect to precision, accuracy, linearity, limit of detection and limit of quantitation. The separation of enantiomers of DXZN, I and II makes the chromatographic method suitable for both qualifying optical purity and isolation of individual enantiomers. It is also useful for determination of chiral impurities during the process development and reaction monitoring during the synthesis of DXZN. The advantages of the developed method include (i) separation of the enantiomers of the synthetic precursors and doxazosin was achieved in a single run, (ii) the observed elution order R(-) < S(+) is beneficial for quantitative analysis, since the minor enantiomer eluted first, assuming that the S(+) enantiomer is the desired one and (iii) as the applied chiral stationary phase is available in bulk, it makes the chromatographic method up scalable for eventual (semi) preparative purification and production of the desired enantiomer (S(+)).

Rabeprazole, 2-[[[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl] methyl] sulfinyl]-1H-benzimidazole is one of latest proton-pump inhibitors developed for suppression of gastric acid secretion by inhibition of the H⁺/K⁺-ATPase [53]. It is one of the most potent therapeutic agents used for treatment of several gastrointestinal disorders such as gastric and duodenal ulcers [54].

Like other proton-pump inhibitors viz., omeprazole, lansoprazole and pantoprazole, it has an asymmetric sulfoxide function resulting in a pair of enantiomers of non-superimposable mirror images. A simple and rapid liquid chromatographic method for enantioselective separation and determination of R-(+) and S(-) enantiomers of rabeprazole in drugs and pharmaceuticals using photo diode array (PDA) and polarimetric detectors connected in series was developed by R. Nageswara Rao et al. [55]. AD-H (250mm×4.6 mm) 5 µm column packed with amylose tris(3,5-dimethylphenyl carbamate) as a stationary phase and the mobile phase containing n-hexane:ethanol:2-propanol(75:15:10, v/v/v) in an isocratic mode has yielded baseline separation with resolution greater than 3.0 at 40°C. Effects of ethanol, 2-propanol and temperature on separation were studied for optimum resolution. Lansoprazole sulphone was used as an internal standard (IS) for quantitative determination of individual enantiomers in bulk drugs as well as pharmaceutical formulations. The method was validated in terms of accuracy, precision and linearity according to ICH guidelines. The linearity of the method was studied in the range of 0.5–50µg/ml and the r^2 was >0.9997. The inter- and intra-day precision of assay were determined (R.S.D. < 1%) and the recoveries were in the range of 99.63–100.22% with <1% R.S.D. The limits of detection (LOD) and quantification (LOQ) were 0.02µg/ml and 0.07µg/ml for both the enantiomers, respectively. The developed method was found to be quite simple, rapid, and sensitive for enantioselective separation and determination of rabeprazole enantiomers in bulk drugs and pharmaceuticals

Biological activity of pharmaceutically some important molecules such as the class of dihydropyrimidine (DHPM), which includes a large number of molecules containing a DHPM ring, is often strictly dependent on chirality. Among DHPMs, several dihydropyrimidinones and dihydropyrimidinethiones (such as Monastrol) have been demonstrated to possess different pharmacological activities depending on their chirality. Identified as leadcompounds for the design of novel anticancer drugs [56], for mode, through an approach in which a chemical modification of the molecules was necessary to overcome solubility limits [57]. Similar experimental limitations are expected also for other DHPMs under analogous chromatographic conditions [58]. G. Nadalini et al. developed high-performance liquid chromatographic separation of dihydropyrimidine racemates on polysaccharide-derived chiral stationary phases. In this study the chromatographic behavior of a set of racemic dihydropyrimidines (DHPMs) has been evaluated on two polysaccharide-derived chiral stationary phases under normal phase conditions [59]. One of these is coated, the other chemically immobilized. The outstanding solvent compatibility of the immobilized chiral stationary phase (CSP) permits the use of solvents such as ethyl acetate (EtOAc) that are unsuitable for coated supports, for which traditional 2-propanol:hexanes mixtures have been employed. Drastic changes in the chromatographic retention and resolution of DHPMs and, in general, in the separation performances have been observed for the two systems. From a practical point of view, EtOAc has been proven to be a better choice for the separation of this important class of compounds. By comparing different molecules different in specific positions of their scaffolds, hypotheses concerning the role of individual chemical groups on retention and selectivity have been done. These effects have been quantified, in some cases, in terms of standard Gibbs energy variations. Even if no chromatographic measurements have been made under nonlinear conditions, clear indications of the potential use of immobilized chiral adsorptive media operated with EtOAc:hexanes mixtures for preparative separations of DHPMs have been evidenced. Explanation of why apparently minor chemical changes to DHPM structures provoked dramatic effects in the enantio-recognition process have been formulated in light of this model. From a practical point of view, the possibility of using EtOAc as MP modifier on immobilized supports has led to a general improvement of chromatographic performances for all of the investigated DHPMs, allowing for a larger number of degrees of freedom in experimental setups. This is reflected by larger separation factors, lower retention and separation of molecules unresolved with 2-propanol:Hex mixtures. Polysaccharide immobilized CSPs operated with EtOAc-based

MPs have been demonstrated to possess important characteristics to be efficient systems for DHPM preparative enantioseparations.

The chiral separation of four stereoisomers of a novel antianginal agent has been investigated on a chiral cellulose column with UV and circular dichroism (CD) detection by M. Beaufour et al. [60] This benzoxathiepin derivative under development has two stereogenic centers whose (R,S) stereoisomer shows an interesting antianginal activity. After optimisation of the mobile phase composition, a baseline-resolved separation of the four stereoisomers was achieved on a Chiralcel OJ-H chiral column by using methanol–ethanol–diethylamine (25:75:0.1, v/v/v) as mobile phase. The CD detection system allowed quantitation and a linear response was observed within a 10–200 $\mu\text{g mL}^{-1}$ concentration range ($r^2 = 0.9966$) and limit of quantification down to 2 $\mu\text{g mL}^{-1}$ was achieved. In addition, the enantiomeric purity of R,S stereoisomer has been quantitatively determined between 1 and 10%. The method described could be further applied to study the pharmacokinetics of the enantiomers of this chiral drug in biological samples and compared with results obtained by capillary electrophoresis [61].

Thioridazine is marketed in the racemic form as the hydrochloride of 10-[2-(-methyl-2-piperidyl) ethyl]-2-methylthiophenothiazine. It belongs to the antipsychotic phenothiazine group and is mostly prescribed for the symptomatic management of psychotic disorder [62]. Thioridazine possesses a stereogenic centre at position 2 in the piperidyl ring. (R)-Thioridazine has a 2.7 times higher affinity than (S)-thioridazine for D2 receptors in isolated rat brain preparation while (S)-thioridazine has a 10 times higher affinity for the D1 receptors [63]. The antipsychotic effect is believed to be associated with (R)-thioridazine [64]. Thioridazine has been resolved into its enantiomers either directly or indirectly by different chromatographic techniques.

R. Bhushan and D. Gupta [65] worked on resolution of racemic thioridazine obtained from Thioril[®] tablets (Cipla Ltd., Goa, India) into its enantiomers by HPLC method involves direct separation using a β -cyclodextrin (CD)-bonded chiral stationary phase at specific pH and mobile phase. The optimum conditions of resolution were established by systematically studying the effect of organic modifier, concentration of buffer, pH and flow rate of mobile phase. The detection limit was found to be 10 μg (5 μg of each enantiomer). The enantiomeric purity of each of the resolved isomers was verified by optical rotation. The chromatographic conditions described herein provide a novel, rapid and reliable approach for separation and analysis of thioridazine enantiomers from commercial samples. Pure sample in quantitative yield from pharmaceutical dosage form can also be obtained. The selectivity and migration behaviour of thioridazine are greatly influenced by interaction of thioridazine with β -cyclodextrin in which inclusion complex formation and hydrogen bonding play a significant role. The newly developed reversed-phase HPLC method provides a separation superior to other HPLC methods. It is a simple method with good linearity, precision and detection sensitivity for separation of thioridazine stereoisomers. Furthermore, this method is applicable and useful for evaluation of thioridazine stereoisomers in pharmaceutical formulations.

Recently, the synthesis of isomers of xanthonolignoids, namely rac-trans-kielcorin C (1), rac-trans-kielcorin D (2), rac-trans-isokielcorin D (3), and rac-trans-kielcorin E (4) was described [66]. The racemates of kielcorins 1–4 demonstrated an inhibitory effect on the *in vitro* growth of different human cancer cell lines and also revealed an effect compatible with protein kinase C (PKC) inhibition similar to that exhibited by the well established PKC inhibitor chelerythrine [67]. The enantioresolution by analytical HPLC using carbamate CSPs of polysaccharide derivatives in multimodal elution was already investigated [68].

The enantiomers of the xanthonolignoids 1–4 were, for the first time, separated by high performance liquid chromatography on a chiral stationary phase in multimilligram scale on tris-3,5-dimethylphenylcarbamate amylose phase using polar organic conditions as mobile phase [69]. The chromatographic method described is robust and after evaporation of the fractions collected the analysis of them did not show any degradation of the solutes. A solid-phase injection system was developed and combined with a closed loop recycling system to increase

the productivity and recovery of the preparative process. The solid-phase injector became a valuable alternative to the traditional sample injector in preparative HPLC for low-solubility samples. The effects of the racemates and the related enantiomers on the *in vitro* growth of human breast cancer cell line MCF-7 were compared. Differences in their growth inhibitory activity were observed. Besides the antitumor activity, these enantiomers of kielcorins will allow the study of the stereochemical influence on other activities.

Stereospecific HPLC method for separation of Frovatriptan enantiomers in bulk drug and pharmaceutical formulations was developed and validated on a normal-phase amylose derivitized chiral column by M. Khan et al [70]. The chiral separation was achieved in amylose carbamate derivitized column (Chiralpak AD-H). The effects of the organic modifiers namely 2-propanol, ethanol and diethyl amine (DEA) in the mobile phase were optimized to obtain the best enantiomeric separation. Calibration curves were linear over the range of 200–6150 ng/mL, with a regression coefficient (R²) of 0.9998. The limit of detection (LOD) and limit of quantification (LOQ) were 65 ng/mL and 200 ng/mL, respectively. The method was accurate and precise and suitable for the intended purpose. Analysis results were compared with the results obtained by using a validated chiral CE method and found to be in very good agreement. This method can be successfully applied to the enantiomeric purity analysis of Frovatriptan in pharmaceutical bulk drug samples and formulations.

Determination of the kinetic and equilibrium parameters of the *n*-boc-rolipram enantiomers on a chiral stationary phase by high performance liquid chromatography considered by C.V. Goncalves et al. [71]. In this study, the overall mass transfer coefficient, diffusivities, axial dispersion coefficient, equilibrium constants of the two enantiomers and bed voidage of the column packed with cellulose tris(3,5-dimethylphenylcarbamate) supported on silica as the chiral stationary phase (CSP) and hexane/2-propanol as the mobile phase for the separation of racemic *n*-boc-Rolipram were determined by moment analysis. The CSP shows a greater affinity for the R enantiomer of *n*-boc-Rolipram than for S enantiomer. The equilibrium and kinetic parameters of the *n*-boc-Rolipram enantiomers support an adsorption dynamics which favors the R-boc-Rolipram separation. Although, both the enantiomers shows a good affinity for the synthesized CSP, the diffusive process of enantiomers through the pores of cellulose tris(3,5-dimethylphenylcarbamate) supported in silica is crucial for the dynamics of the mass transfer. The results show that diffusion dynamic plays an important role on the separation process of the both enantiomers. These parameters are very important for the design of continuous preparative chromatography like simulated moving bed (SMB).

M.M. Hefnawy et al. [72] developed a sensitive and selective high-performance liquid chromatographic (HPLC) method for the simultaneous determination of bufuralol enantiomers in plasma and pharmaceutical formulations. Enantiomeric resolution was achieved on a vancomycin macrocyclic antibiotic chiral stationary phase (CSP) known as Chirobiotic V with UV detection set at 254 nm. The polar ionic mobile phase (PIM) consisting of methanol–glacial acetic acid–triethylamine (100:0.015:0.010, v/v/v) has been used at a flow rate of 0.5 mL min⁻¹. The method is highly specific where other coformulated compounds did not interfere. The stability of bufuralol enantiomers under different degrees of temperature was also studied. The results showed that the drug is stable for at least 7 days at 70 °C. The method was validated for its linearity, accuracy, precision and robustness. An experimental design was used during validation to evaluate method robustness. The calibration curves in plasma were linear over the range of 5–500 ng mL⁻¹ for each enantiomer with detection limit of 2 ng mL⁻¹. The mean relative standard deviation (RSD) of the results of within-day precision and accuracy of the drug were ≤10%. There was no significant difference (*p* > 0.05) between inter- and intra-day studies for each enantiomer which confirmed the reproducibility of the assay method. The mean extraction efficiency for S-(–)- and R-(+)-bufuralol from plasma was in the range 97–102% at 15–400 ng mL⁻¹ level for each enantiomer. The overall recovery of bufuralol enantiomers from pharmaceutical formulations was in the range 99.6–102.2% with %RSD ranging from 1.06 to

1.16%. The assay method proved to be suitable as chiral quality control for bufuralol formulations by HPLC and for therapeutic drug monitoring.

W. Lindner et al. [73] considered novel strong chiral stationary phase for the enantiomer separation of chiral amines. They developed and described successful brush-type CSPs for the enantiomer separation of chiral amines in HPLC acting as a cation exchange material based on the novel designed synthetic low molecular mass selectors (SOs) in concerned with additional intermolecular interactions. The SO as the functional unit for enantioselectivity contains a β -aminocyclohexanesulfonic acid moiety and is readily accessible via straightforward synthesis in both enantiomeric forms yielding chiral stationary phases (CSPs) with opposite configurations (Fig. 3), CSPs 1 and 2, and reversed elution orders.

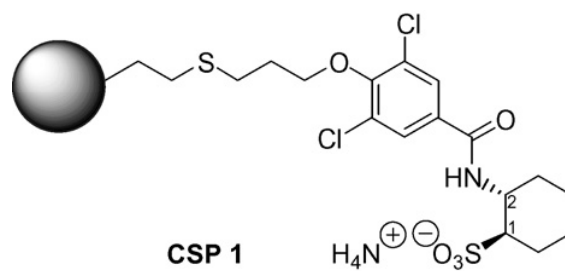


Fig. 3. Chemical structure of novel chiral strong cation exchanger material CSP 1 being of (1R,2R)-configuration. CSP 2 is of opposite configuration.

Chromatographic evaluation of the novel CSPs was carried out in nonaqueous polar organic mobile phase conditions where detailed investigations of the operating conditions in terms of proton activity, type of co- and counterion, concentration of the counterion, and type of bulk solvent were accomplished. Thereby, ion pairing and ion exchange was determined as the dominant process for solute retention and insights into the mechanism of enantiodiscrimination of CSPs 1 and 2 were provided. Furthermore, generally optimized mobile phase conditions were established and applied for a larger set of drug analytes. The findings presented here are promising and demonstrate the potential of enantioselective strong cation exchanger type CSPs based on synthetic low molecular mass SOs. A dedicated program to further elucidate the future of cation exchanger materials with improved properties in terms of selectivity and applicability ranges is in progress.

5. Conclusions

We have reviewed evolution in the design and synthesis various types of chiral stationary phases which mainly belonging to the hydrogen bonding, π interaction and ligand metal complexation type for HPLC, and shown these phases were very efficient for the direct resolution of a wide range of racemic compounds including many chiral drugs. Some of these phases are now commercially available and they are very effective for the routine analysis of various chiral compounds. Each CSP has its own characteristic feature of chiral recognition and has led to new materials with overall enhanced properties in terms of enantioselectivity and broad applicability at both the analytical and preparative scales.

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