Enantioselective chromatography in drug discovery

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Molecular chirality is a fundamental consideration in drug discovery, one necessary to understand and describe biological targets as well as to design effective pharmaceutical agents. Enantioselective chromatography has played an increasing role not only as an analytical tool for chiral analyses, but also as a preparative technique to obtain pure enantiomers from racemates quickly from a wide diversity of chemical structures. Different enantioselective chromatography techniques are reviewed here, with particular emphasis on the most widespread high performance liquid chromatography (HPLC) and the rapidly emerging supercritical fluid chromatography (SFC) techniques. This review focuses on the dramatic advances in the chiral stationary phases (CSPs) that have made HPLC and SFC indispensable techniques for drug discovery today. In addition, screening strategies for rapid method development and considerations for laboratory-scale preparative separation are discussed and recent achievements are highlighted.

Living organisms are based on a plethora of chiral molecules and often display different biological responses to drug enantiomers when they are dosed separately. It is not uncommon for one enantiomer to be active while the other is toxic in biological systems [1,2]. Thus, the FDA has required evaluation of each enantiomer in developing stereoisomeric drugs [3]. As a result, the pharmaceutical industry has raised its emphasis on the generation of enantiomerically pure compounds before undertaking pharmacokinetic, metabolic, physiological and toxicological evaluation in the search for drugs with greater therapeutic benefits and low toxicity [4,5]. According to the recently survey by Israel Agranat [6], the distribution of worldwide approved drugs from 1983–2002 and FDA-approved drugs from 1991–2002 indicate that single-enantiomers surpassed achirals whereas racemic drugs represented the minority category. The distribution of the 15 FDA-approved drugs in the period of January-August 2003 are 64% single enantiomers, 14% racemates and 22% archials [6].

Although a large number of approaches have been used to isolate single enantiomers [2,7], enantioselective chromatography using HPLC and SFC on chiral stationary phases (CSPs) has become the most widely utilized technique in the context of obtaining limited quantities (from mg to multi-grams) of pure enantiomers quickly, particularly in drug discovery [8]. The impetus for reliance on chromatography has been fueled by recent advances in CSPs that allow reliable, robust and efficient resolution of mg to gram quantities of chiral molecules in a matter of hours. At the drug discovery stage, minimizing the time required to obtain pure enantiomers is of paramount importance. In addition, enantioselective chromatography usually furnishes both enantiomers with the high enantiopurity that is required for comparative biological, pharmacological or toxicological evaluations [9,10].

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Direct chromatographic resolution using CSPs is preferred to indirect approaches, such as derivatization [11] or use of chiral additives in the mobile phase, because much less sample manipulation is required, and more rapid solute recovery is possible after the preparative chromatography. This review will only examine the direct enantioselective chromatography on CSPs that is most frequently used in drug discovery.

Techniques of enantioselective chromatography on CSPs

The techniques of enantioselective chromatography on CSPs can be broadly classified into the following five categories [12].

Gas chromatography (GC)

GC remains a powerful analytical chiral separation technique [13,14], thanks to the development of new CSPs with better thermal stability and the advances in capillary column technology. GC offers advantages such as high resolution, superior column efficiency and simple mobile phase composition. It is particularly useful for the resolution of nonaromatic compounds used in asymmetric syntheses, which are not easily separated and detected by liquid chromatography (LC). However, GC is limited to compounds that are thermally stable and volatile, either in their native state or following derivatization. In addition, CSPs can racemize at high temperatures, and this typically results in a decrease in the separation factor (α, the enthalpy difference). Moreover, it is very difficult to scale up capillary GC enantioseparations for a meaningful preparative separation in the drug discovery environment.

Capillary electrophoresis (CE)

Within the past decade, CE has been established as a powerful technique for analytical enantioseparation, based on its high resolution due to plug flow and its flexibility with regard to separation conditions. In CE, instead of CSP, a chiral selector is usually used as a running buffer additive forming a so-called pseudophase. Whereas analytes and chiral selectors both possess electrophoretic mobility in the same phase, the chiral separation based on their enantioselective interactions can be described as 'capillary electrokinetic chromatography' which was introduced by Terabe *et al.* [15]. CE has been successfully applied in the separation of a wide variety of chiral molecules [17,18]. The success of CE for pharmaceutical and biomedical analysis has prompted the introduction of CE into the US Pharmacopeia and European Pharmacopoeia [16]. However, CE, like GC, relies on the use of narrow capillaries to generate their high separation efficiencies. Therefore, it is generally impracticable for preparative-scale separations. Consequently, during the early stage of drug discovery, the potential requirement to obtain mg to multigram quantities of pure enantiomers for a new chiral molecule often excludes CE as a primary separation technique.

Thin-layer liquid chromatography (TLC)

TLC is a useful technique that entails minimal cost with limited resolution and precision [19]. It has not been widely used for chiral separations, and will remain underutilized until more inexpensive chiral TLC plates with high selectivity or wide applicability for chiral resolution become commercially available.

High performance liquid chromatography (HPLC)

HPLC on CSPs has developed dramatically in the past two decades [20,21]. It is the most widespread chiral separation technique used in drug discovery. This is not only related to the common availability and demonstrated success of HPLC for performing separations in the pharmaceutical industry, but also because HPLC-CSPs can be used both for analytical and preparative chiral resolution in a very efficient and flexible way. The technique allows effective scale-up to the amounts of material needed in the different phases of drug discovery simply by increasing the column size and by using throughput-improving techniques [22,23] in laboratory-scale batch chromatography (up to 100g), or using simulated moving bed (SMB) methods [24] in process-scale chromatography (100g to Kgs of pure enantiomers).

Supercritical fluid chromatography (SFC)

In recent years, the rapidly emerging technique of supercritical fluid chromatography (SFC) with packed-CSP-columns has reached a new level of utility in drug discovery [25], as evidenced by the steady rise in the number of industrial users as well as the dramatic increase in instrument sales. SFC, which uses a supercritical or near critical fluid, such as CO_2, often provides a three to five times faster separation than normal-phase HPLC due to its higher diffusivity (i.e. higher optimal linear velocity) and lower viscosity (i.e. lower pressure drop) [27]. Such great speed advances are extremely important not only for analytical separation, but even more so for preparative enantioseparations in today's drug discovery environment. As commercial SFC instruments continue to improve, SFC is becoming the first choice in enantioseparation and purification due to its significant and intrinsic benefits including:

- Higher resolution per unit of time, faster column re-equilibration and simpler mobile phase composition, resulting in faster method development, analyses and purification when compared with HPLC.
- Unique ability to vary mobile phase strength by controlling its density through pressure and temperature.
- Complementary to HPLC with its ability to exploit different selectivities because the mobile phase interactions are not equivalent between HPLC and SFC, and compatibility with practically all chiral selectors used in HPLC and GC.
- Greater solvent compatibility of CO_2 with polar solvents compared with hexane, providing more flexibility in mobile phase and solvent selection.

TABLE 1

Types of CSPs and their common loading capacities [8,10]

Type	CSPs	Common loading capacity (mg solute / g CSP)
I	Pirkle-type (π-Complex)	1–50
II	Polysaccharide derivatives	5–150
III	Macrocyclic-type	
	Native and derivatized Cyclodextrins	0.1–5
	Glycopeptides	0.1–5
	Chiral crown ether	0.1–5
IV	Ligand exchange	0.1–1
V	Proteins	0.1–0.2
VI	Other polymer type	1–100

- Higher preparative chromatography production rate, lower organic solvent usage and removal, and environmental friendliness of CO_2.
- A combination of the inherent advantages of GC without the limitation of high temperature on enantioselectivity. Compatible with both HPLC and GC detectors.

As the most widespread and rapidly emerging chiral chromatography techniques in drug discovery, enantioselective HPLC and SFC on CSPs will be the focus of this review.

HPLC and SFC CSPs under different mobile-phase modes

Major developments in chiral stationary phases have led to dramatic advances in enantioselective chromatography [28]. HPLC–SFC enantioseparation on CSPs results from energy differences between transient diastereomeric complexes formed by the solute-CSP interactions. These differential interactions are directly influenced by the mobile-phase environments. In addition to the normal and reversed phases referenced in other forms of LC, a third mode, polar organic mode, was identified by Armstrong in 1992 during his work on cyclodextrin CSPs [29]. In this mode, addition of a polar organic solvent (such as alcohol) to a non-polar solvent (such as acetonitrile) mediates hydrogen-bonding and dipole–dipole interactions at the mouth of the cyclodextrin molecule, whereas acetonitrile suppresses the formation of an inclusion complex. This terminology is now also used by others to describe a combination of polar alcohols that results in modified inclusion and simultaneous control of hydrogen-bonding effects. A fourth mode, polar ionic mode, applies when ion-exchange sites are present in the CSP and the mobile phase contains a volatile salt (or an acid and a base) to control the ion-exchange function as well as a polar alcohol to mediate hydrogen bonding. Recent developments in chiral columns are focused on multi-modal CSPs for broader applicability.

It is estimated that 1300 CSPs have been prepared, and over 200 CSPs have been commercialized [30]. Thus, understanding and classifying the different CSPs is important for selecting the most suitable CSP to solve a particular problem. The most commonly used CSPs for HPLC and SFC are grouped into six categories in Table 1.

Pirkle-type CSPs

Pirkle-type CSPs require π–π interaction between the CSP and solute as well as other simultaneous interactions including hydrogen bonding and dipole stacking. Because these interactions are favored in non-polar solvents, Pirkle-type CSPs are generally used in the normal-phase mode (NP), although reversed-phase separations can also be performed using these CSPs, particularly for ionic and highly polar compounds, such as carboxylic acids. Indeed, the first reported packed-column SFC enantioseparation used a Pirkle-type CSP [31]. Since then, these CSPs, especially the newer polysiloxane-based columns (poly-Whelk-O) [32], have demonstrated great selectivity, high efficiency and reduced retention in SFC. Recently, ZirChrom Separations (Anoka, Minnesota, USA) has announced a series of Pirkle-type π-electron ligands in their pH-stable zirconium-based columns. These ligands are bonded to porous and non-porous particles [33]. Although a substantial reduction in analysis time was demonstrated using the non-porous structure, such a column can compromise the sample loading and yield high backpressure.

Derivatized polysaccharide CSPs

Okamoto, *et al.* were the first to coat polysaccharide derivatives successfully onto macroporous γ-aminopropyl silanized silica [34]. The resulting stable and highly efficient CSPs, now trademarked as Chiralcel and Chiralpak (Daicel Chemical Industries) for cellulose and amylose derivatives, respectively, are the most popular CSPs in use today. They dominate the overall applications in the pharmaceutical industry because of their versatility and generally high loading capacity.

The derivatized polysaccharides are multi-modal CSPs. In normal-phase mode HPLC or in SFC a different alcohol or concentration of alcohol often results in different enantioselectivities. Solid-state NMR studies examining the effect of alcohols on a given CSP demonstrated the alterations in the steric environment of the chiral cavities by different alcohols (branched and linear) or by different concentrations of the same alcohol [35]. Although branched alcohols (such as t-butanol) tend to provide higher selectivity and short linear alcohols often show higher efficiencies, the modifier effects cannot be generalized as evidenced by the fact that just as many compounds were separated using small alcohols as were separated using large ones. Such modifier effects on selectivity are more dramatic on the polysaccharide CSPs than on other types of CSPs, which is a benefit that increases the likelihood of achieving a specific enantioseparation. These CSPs can also be used in the reversed-phase mode, which is preferable for more-polar compounds or biological molecules. The polar organic mode using either a binary acetonitrile–alcohol or alcohol–alcohol mixture is very popular on these CSPs, often

providing excellent resolution with shorter retention in addition to offering a wider solubility range.

The derivatized polysaccharide CSPs are extremely successful in SFC. In addition to the aforementioned advantages of SFC, these CSPs do not need acidic additives when used with SFC [36], thus reducing the risk of esterification during work up. This benefit can be attributed to the somewhat acidic nature of CO_2 due to the trace level of water or the proton donor from an alcohol modifier. Furthermore, amine additives do not remain after their removal from the CO_2 mobile phase in SFC [37], in contrast to the strong memory effects of base or acid additives in HPLC as demonstrated by Stringham *et al.* [38,39]. Recently, Stringham reported increasing retention and selectivity in SFC by incorporating cyclic amines (larger than cyclobutyl) into the modifier, to accomplish SFC separation of amphetamine and methamphetamine enantiomers in five minutes [40], as shown in Figure 1.

The main drawback of these polysaccharide CSPs is the fact that they are coated onto silica gel and thus are only compatible with a limited choice of solvents. This year, Chiral Technologies, Inc. (Daicel) has introduced Chiralpak IA, an immobilized amylose tri(3,5-dimethylphenyl carbamate derivative CSP column, which is produced via a proprietary process. This first commercialized bonded-type CSP shows greater durability in all organic solvents and allows a much wider range of mobile phase selections to optimize enantioselectivity, as well as solute dissolution. However, because any bonding process can cause structural alteration of the polysaccharides, Chiralpak IA might show different selectivity from its coated-type counterpart, Chiralpak AD, under the same mobile phase conditions.

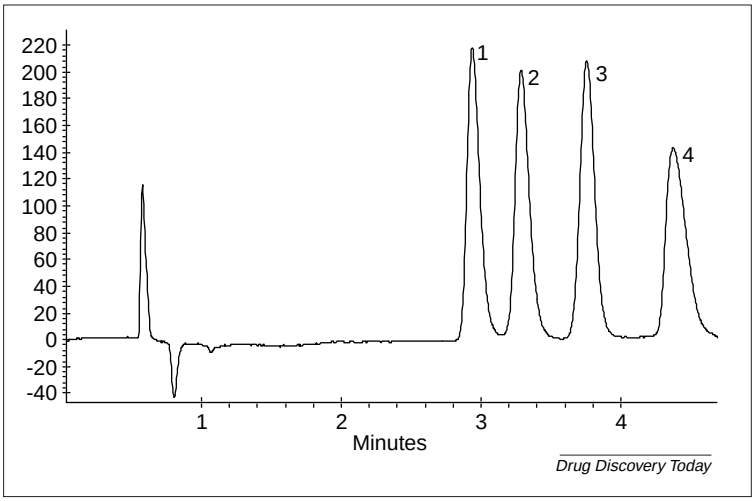

FIGURE 1

SFC separation of amphetamine and methamphetamine enantiomers on a CHIRALPAK® AD-H® column using a 10% 2-propanol (0.5% cyclohexylamine) modifier at 5 ml/min; 150 bar. Peak 1, (*S*)-methamphetamine, 2.94 min; peak 2, (*R*)-methamphetamine, 3.28 min; peak 3, (*S*)-amphetamine, 3.75 min; peak 4, (*R*)-amphetamine, 4.37 min. (Permission granted by R.W. Stringham [40])

Macrocyclic-type CSPs

Cyclodextrins (CDs), glycopeptides and chiral crown ethers are the three main types of macrocyclic CSPs. Although CDs and glycopeptides are the most utilized chiral selectors in CE, and CDs dominate chiral GC, these macrocyclic-type CSPs have not been the top choices in HPLC and SFC. Nevertheless, they represent an important type of HPLC–SFC CSP.

Since the first commercialized CD-bonded CSP was introduced in the reversed-phase mode by Armstrong *et al.* [29], a wide variety of derivatized CDs have been developed as multi-modal CSPs. The most widely used and effective CSPs are the hydroxypropyl-β-CD (Cyclobond I RSP) and the aromatic derivatized CD such as Cyclobond I DMP and Cyclobond I RN or SN (Advanced Separation Technologies). In reversed-phase mode, retention is mainly due to hydrophobic inclusion with hydrogen-bonding and steric interactions at the mouth of the cavity. Structural features of the solute, such as aromatic ring systems at the α or β position with comparable size to the cyclodextrin cavity and at least one hydrogen-bonding group near the chiral center, are important for achieving enantioselectivity. In the polar organic mode (acetonitrile–alcohol mixture), the chiral compound is retained via a combination of hydrogen bonding and dipole–dipole interactions at the mouth of the CD. Thus, a minimum of two separate hydrogen-bonding groups (one of which resides on or near the chiral center) and a bulky moiety close to the chiral center are necessary for the enantioselectivity. Cyclobond 1 RN and SN CSPs also work well in normal-phase mode as well as in SFC because the aromatic CDs can be used for π-complexation. More recently, YMC (Kyoto, Japan) has offered native and permethylated β CDs for preparative application, and Shiseido has introduced a phenylcarbamated β CD CSP [41].

Since the first introduction of glycopeptide CSPs by Armstrong *et al.* in 1994, four types of CSPs have become commercially available. Immobilized vancomycin, ristocetin, teicoplanin and teicoplanin aglycon CSPs are marketed by Advanced Separation Technologies as Chirobiotic V, R, T and TAG, respectively. These four CSPs have proven to be complementary in separating a broad range of chiral molecules in all four mobile phase modes [42]. One most noticeable advantage of the glycopeptide CSPs is that native, underivatized amino acids can be separated effectively with a simple alcohol–water mobile phase [43,44]. The macrocyclic glycopeptide CSPs have also been widely used in SFC recently [45,46]. Generally speaking, the teicoplanin aglycon and teicoplanin CSPs seem to have greater enantioselectivity than ristocetin A and vancomycin CSPs. It is also worth noting that the Chirobiotic columns have been used extensively to separate chiral and achiral metabolites by LC-MS in recent years due to their unique ability to separate polar molecules.

Chiral crown ether CSPs are powerful tools for the separation of chiral molecules containing primary amino groups

in the reversed-phase mode with an acidic mobile phase. In this class of CSP, chiral recognition is based on formation of an inclusion complex between the primary amine group (in the ammonium ion form) and the chiral crown ether. A new generation of chiral crown ether-based CSPs employing a covalently-bound (+) or (–) tetracarboxylic acid has been developed by Rstech Corp. (Daejeon, Korea) and has been introduced commercially by Regis Technologies. These bonded CSPs allow an organic modifier, such as methanol, to be used with a variety of acids to reduce retention and improve efficiency. Contrary to the common assumption that only primary amines can be resolved, secondary amines including β-blockers have been successfully resolved by Steffeck, *et al.* [47]. With these new findings, an expanded use of crown ether-based CSPs can be expected in the future.

Ligand exchange-type CSPs

Ligand exchange CSPs consist of a chiral bidentate ligand immobilized on the column. To achieve enantioseparation, a chiral molecule must be able to form coordination complexes with a transition metal ion such as copper (II) present as an additive in the aqueous mobile phase. These CSPs provide excellent enantioresolution for amino acids, amino acid derivatives, hydantoins and amino alcohols. The compounds do not require UV chromaphores to be detected by a UV detector as the copper complexes absorb in the UV region. Detection sensitivity, however, is compromised due to the background signal.

Protein-based CSPs

Protein-based CSPs have broad enantioselectivity for analytical chiral separations. However, in recent years the application of these CSPs has declined because they are less stable and more expensive than most other CSPs, and they can only be used in reversed-phase mode. In addition, protein-based CSPs are not practical for preparative applications because they exhibit the lowest loading capacity of all CSPs.

Other polymer-type CSPs

Other chiral polymers including crosslinked diallyltartrinine amide (Kromasil CHI) and polyacrylamide (Chiraspher) are also among the most successful CSPs, especially for preparative chiral resolution of drug molecules.

Strategies for fast enantioselective method development in drug discovery

Enantioselective chromatography has advanced dramatically in the past two decades, led by the development of new CSPs. However, the demand for faster preparative and analytical chiral resolutions for a wide variety of new chemical entities continues to push the frontiers of current technologies. As there is no universal CSP, elucidation of the chiral recognition mechanisms operating at the molecular level is essential for further development in the field. Currently, prediction of enantioselectivity for almost any CSPs is, practically speaking, still not feasible. In addition, small changes in solute structure and/or chromatographic environment often have great impact on the chiral resolution ability of many CSPs. Consequently, 'trial-and-error' screening of a set of CSPs that offers a broad-spectrum of enantioselectivity in simple mobile phase systems has been the most popular approach to chiral method development in drug discovery.

To facilitate this approach, three automated column screening strategies have been reported. The most routinely used approach [48,49] employs automated column and solvent switching to screen the columns or mobile phases one at a time on a 24-h basis. This simple approach has been incorporated in commercial instruments, but it is slow because each column has to be equilibrated and tested individually. Another approach is to couple columns in series as proposed by Wang, *et al.* [50] using CHIROBIOTIC columns. Three different short columns were connected in series and screened as one column using different mobile phases. Although fast, this technique cannot be used to screen mixed-type CSPs or the same-type CSP (such as polysaccharide-derivatives) on which elution order reversal between columns is common [51]. Even on CHIROBIOTIC columns, contrary to initial beliefs, elution order reversal has been observed [52]. Moreover, screening with columns coupled does not identify the best column to use. Deconvolution is necessary using the selected mobile phase, which increases the screening time and makes it more difficult to automate the entire process.

Very recently, a multi-column parallel screening approach with a circular dichroism signal pooling technique was reported [53]. Five CSPs were screened simultaneously in parallel using a simple customized HPLC system with five UV detectors and one circular dichroism detector. An injected sample was carried by the mobile phase through an on-line pre-filter, then divided into five columns and UV detectors with even flow distribution accomplished via individually adjusted backpressures. The five channels after the UV detectors were recombined using a reverse splitter into the circular dichroism detector. As shown in Figure 2, the enantioselectivity of sulcoazole was screened on five CSPs in parallel resulting in a fivefold increase in throughput. In addition, the circular dichroism signal pooling technique facilitates peak tracking as well as the determination of the sign of chirality of the resolved enantiomers for multiple columns simultaneously.

Considerations of laboratory-scale preparative enantioseparation in drug discovery

The goals of a separation change when moving from an analytical to a preparative scale. Apart from sufficient enantioselectivity, a high loading capacity is an important prerequisite in preparative separation. Although column loading varies with the compound structure and the mobile phase, it is fundamentally related to the number of

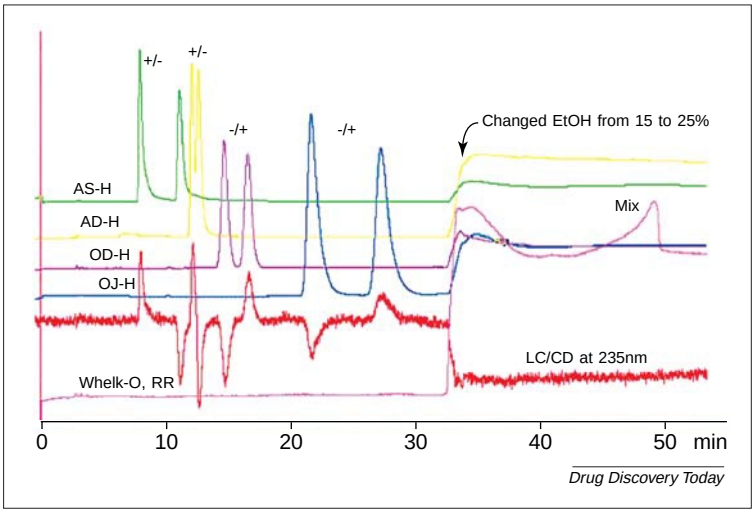

FIGURE 2

Parallel chiral column screening chromatograms of Sulconazole with CD signal pooling. Mobile phase: 15% ethanol in *n*-hexane from 0–30 min and 25% ethanol in *n*-hexane from 30–55 min. Flow rate: 1 mL/min for each column. UV wavelength: 225 nm, CD wavelength 235 nm. (The work of this author [53])

accessible binding sites per unit mass. The common loading capacity is listed in Table 1 for each type of CSP [54]. The polysaccharide-type has the highest loading followed by the cross-linked diallyltartrinine amide (Kromasil CHI) and polyacrylamide (Chiraspher) polymer-type CSPs. Most of the Pirkle-type CSPs also have good loading capacity. In addition, they offer the opportunity to reverse the elution order, such that preparative separations can be carried out favorably. Macrocylic-type CSPs are used less frequently and mostly for resolving mgs of racemates. Ligand exchange and protein-type CSPs are not useful in preparative chiral separation due to the additional steps required to remove metal ion and the very low capacity, respectively.

In preparative separation, selectivity is not always the main criterion for finding the optimal mobile phase. Compound solubility and stability in the mobile phase is equally crucial with respect to the yield of the separation. In addition, a mobile phase that is less expensive, easy to remove and more environmentally-friendly has great benefits. This is consistent with the increasing popularity of SFC and pure polar organic mobile phase in HPLC or simulated moving bed (SMB) chromatography. Furthermore, some successful analytical HPLC mobile phases cannot be used for preparative applications due to their special hazards or difficulties. For example, perchloric acid, widely used on the analytical scale with the crown-ether type CSPs, becomes an explosion hazard during solvent evaporation in preparative applications. Also the transition metal ions in the mobile phase make the ligand-exchange type CSP even less attractive for preparation separations.

Combining short run time and fast solvent removal, SFC is increasing become the method of choice in drug discovery where time constraints are crucial, and it continues to extend the upper limit of a laboratory-scale preparative enantioseparation to a few hundred grams. SMB is also of increasing interest in the pharmaceutical industry as it combines the effective use of expensive CSPs with significant reduction in overall solvent consumption in the production of large amounts of pure enantiomers. By combining SFC's ability to change solvent strength via pressure control with the continuous isocratic SMB process, the SFC–SMB [55], which is essentially a gradient, has great potential to be a highly valuable technique for producing process or production scale enantiomeric drug molecules in the pharmaceutical industry.

Conclusion

Enantioselective chromatography, particularly HPLC and SFC on CSPs, has become an indispensable part of drug discovery not only for chiral analyses but also for the fast preparation of drug molecules. With all of the aforementioned advantages, enantioseparation by SFC is of increasing importance in drug discovery. There is no doubt that preparative chromatographic resolution has become a highly competitive technique for obtaining pure enantiomers in drug discovery. An increase in the availability, robustness and use of SMB or SFC–SMB will potentially boost the exploitation of enantioselective chromatography into drug development and production. As more chiral selectors are being tested and commercialized as CSPs, a better understanding of the chiral recognition mechanisms at the molecular level is essential for the future, so that one day chromatographers can rapidly select or even create suitable CSPs for efficient resolution of specific enantiomers.

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