Journal of Chromatography A, 1217 (2010) 1134–1138

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Systematic evaluation of new chiral stationary phases for supercritical fluid chromatography using a standard racemate library

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ARTICLE INFO

Article history:
Available online 8 October 2009

Dedicated to Professor Volker Schurig, Universität Tübingen, on the occasion of his 70th birthday.

Keywords:
Chiral stationary phase
SFC
Racemate library

ABSTRACT

A systematic approach to the evaluation of new chiral stationary phases (CSPs) for supercritical fluid chromatography (SFC) using a standard library of racemic analytes is described. A standard library of racemic analytes representing a variety of functional group classes was assembled from a mixture of proprietary and commercial compounds. The library is dispensed and stored in a convenient 96-well microplate format to facilitate ease of use, and to minimize the amount of analyte required for analysis. Automated SFC screening was performed on both established CSPs in common use, as well as a group of six recently commercialized CSPs. Screening results were archived in a structure-searchable database that allows convenient comparison of performance data to determine which CSPs shows the best performance.

1. Introduction

Chiral chromatography is an invaluable tool for carrying out analysis in support of pharmaceutical discovery and development. In this technique, the two enantiomers of a chiral analyte are chromatographically resolved using a chiral stationary phase (CSP). Supercritical fluid chromatography (SFC) is a proven technology that is often used to achieve fast chiral separation [1,2]. Many new CSPs are introduced to the market each year [3–7], often with the promise of improved performance relative to existing products. Frequently, evaluation of new CSPs is somewhat haphazard and incomplete, making it difficult to determine with certainty which CSPs are the most valuable, and which show a better performance when compared to existing phases.

This general problem has been recognized in the past and has been addressed by a number of researchers, including Akin et al. [8] who proposed an orthogonal approach for chiral method development screening based on the use of a representative racemate library to screen by various separation modes (normal phase HPLC, reversed phase HPLC, SFC, *etc.*). Using a similar approach, Armstrong and co-workers [9] have evaluated and compared various CSPs using a standard racemate library approach, using an innovative bar graph representation to conveniently visualize the results. The library contains representative drug related compounds that include both commercial and proprietary structures. We herein describe the creation of a comparably simple and straightforward standard library-based screening approach for systematic evaluation of CSPs. This approach allows convenient screening of experimental outcomes using a structure-searchable database to track and compare evaluation results.

2. Experimental

2.1. Chemicals

Bone dry grade carbon dioxide was obtained from Air Gas (New Hampshire, USA). Methanol, 2-propanol (HPLC Grade) and isobutyl amine were purchased from Sigma–Aldrich (St. Louis, MO, USA). 19 commercial racemic compounds that were used for the racemic compound library were trans stilbene oxide,1,2,4,5-tetra-*t*-butylbenzene, mianserin, thalidomide, 1-1′ binaphthol 2,2′ diamine, propanalol hydrochloride, 6-methoxy alpha methyl 2-naphthane acetic acid, lansoprazole, flurbiprofen, flavanone, 2,2,2 trifluoro 1-9 anthrylethanol, warfarin, ibuprofen, indanol, hydrobenzoin, troger's base, methyl mandalate, NEA acetamide and benzoin, which were all purchased from Sigma–Aldrich (St. Louis, MO, USA). In addition, 29 proprietary Merck compounds synthesized in-house were also used in this study.

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doi:10.1016/j.chroma.2009.10.004

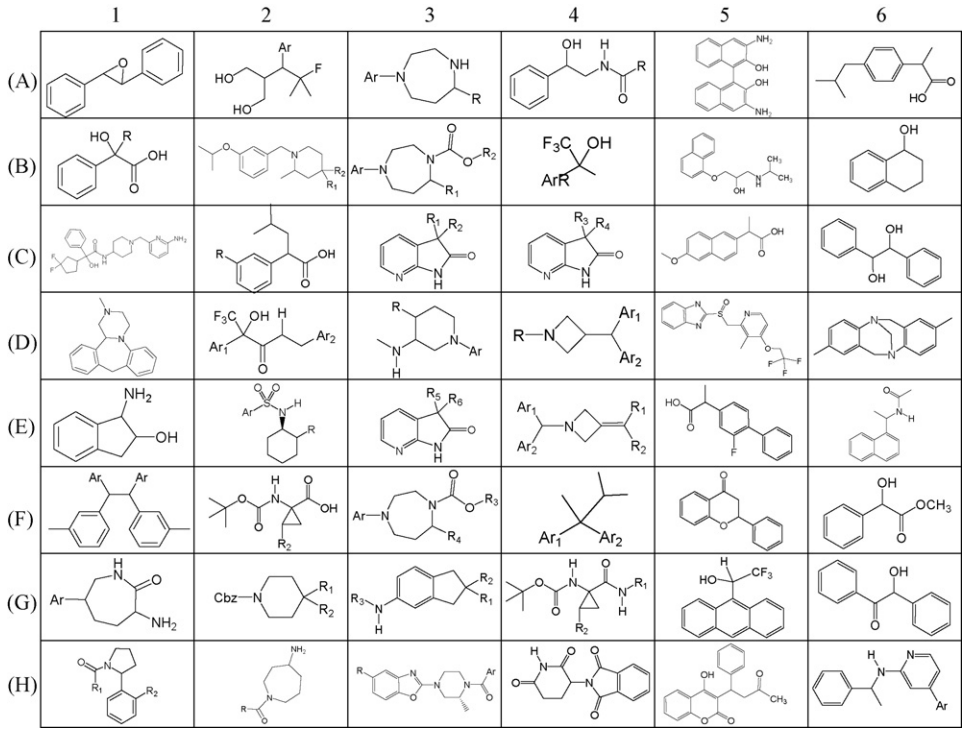

Fig. 1. 48 commercial and proprietary samples from a variety of compound classes were included in the study.

2.2. Chiral stationary phases (CSPs)

Columns packed with Chiralpak AD-H, Chiralcel OD-H, Chiralpak IC were purchased from Chiral Technologies (West Chester, PA, USA). Other columns that were evaluated were RegisCell from Regis Technologies (Morton Grove, IL, USA), Kromasil Cellucoat from Eka Chemicals (Brewster, NY, USA), and Sepapak-2, Sepapak-3 and Sepapak-4 from Sepaserve (Muenster, Germany). All the columns were 25 cm long with 4.6 mm internal diameter and 5 μm particle size, except for Kromasil Cellucoat which has 3 μm particle size.

2.3. Standard racemate library microplates

A stock solution of each racemic compound was prepared by dissolving roughly 1 mg of the compound in 1 mL of 2-propanol or methanol to achieve an approximate concentration of 1 mg/mL. These stock solutions were added to 8 well troughs and a 8× handheld multipipetter was used to transfer ~150 μL of the homogeneous solution of each compound to the wells of a number of racemate library polypropylene microplates according to the layout shown in Fig. 1. Two sets of the 48 racemic compounds were added to each 96-well microplate, with 180° rotation of the plate allowing access to a clean and unused copy of the 48 compound library (Fig. 1). The 96-well microplate was then evaporated to dryness using a Genevac evaporation system. The dried plates were sealed with piercable 96-well microplate cover and stored in a freezer.

Before using a racemate library microplate from the freezer, the microplate was allowed to warm to room temperature. The cover was then removed and the wells were reconstituted with 150 μL methanol or 2-propanol. The plate was then gently vortexed to make homogeneous solutions.

2.4. Instrumentation

The SFC instrument used on the studies was a Berger Analytical from Mettler-Toledo (Delware, USA). The instrument was equipped with a fluid delivery module (a liquid CO_2 pump and a modifier pump), an autosampler with capability to handle 96-well plates, a column oven with a 6-column selection valve, a 6 solvent switching valve, an automatic backpressure regulator, a photodiode array UV detector, and Pronto™ software for instrument control and data processing.

2.5. Chromatographic screening protocols

The 48 samples in the racemic compound library were screened on the 8 chiral stationary phases using standardized SFC method conditions as described in Table 1. For each enantioseparation the resolution was collected using the USP method [10].

3. Results and discussion

When faced with the challenge of developing a standard racemate library for CSP screening, a number of different factors were taken into consideration. First, we reasoned that the compounds in

Table 1
Experimental conditions for chiral SFC column evaluation.

Column	8 columns: Chiralpak AD-H, Chiralcel OD-H, Chiralpak IC, RegisCell, Sepapak-2, Sepapak-3, Sepapak-4, Kromasil Cellucoat
Mobile phase	CO_2/MeOH with 25 mM IBA
Temperature	35 °C
Outlet pressure	200 bar
Gradient	4% MeOH with 25 mM IBA/CO_2 for 4 min then ramp at 4%/min to 40% hold for 2 min at 40%.
Analysis time	The single run cycle time is ~16 min in total with 15 min for the gradient run and ~1 min for sample injection. Screening all 48 samples in the racemic compound library on 1 column required ~13 h of instrument time.
Flow rate	2.0 mL/min
Detection	UV detection at 215 nm
Injection volume	10 μL

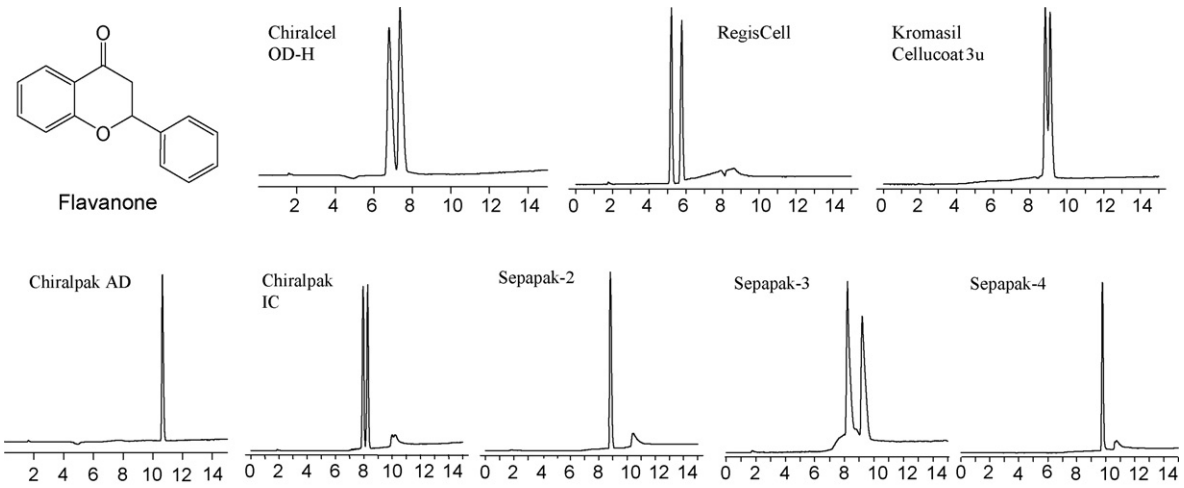

Fig. 2. Comparison of enantioseparation of flavanone on the generic OD-H columns and the Chiralpak AD-H plus five other newly commercialized CSPs.

the library should have a broad range of structural and functional group diversity that would resemble the types of compounds that are typically encountered in pharmaceutical process research. Second, we reasoned that the number of compounds in the library should be big enough to give a sense of the generality of the CSP, but not so big that it would take too long to carry out the evaluation. Finally, we reasoned that the compounds in the library should be readily available in reasonable quantity and purity, and also somewhat stable, so the compound library could be stored and repeatedly used over time.

Based on these criteria, a mixture of 48 commercial racemates and proprietary development compounds were selected, as shown in Fig. 1. This compound set includes neutral, acidic, and basic compounds from a variety of structural classes (alcohols, amines, amides, *etc.*) that are representative of the compounds typically encountered in pharmaceutical process research. Solutions of the compounds (~1 mg/mL in 2-propanol or methanol) were dispensed to several dozen 96-well microplates using handheld 8× multipipetters, and the plates were evaporated to dryness. Each 96-well microplate includes two sets of the 48-member library, so that accessing a fresh and unused library simply involves a 180° rotation of the microplate. The microplates thus prepared were sealed with capmats and stored in a freezer, and have remained suitable for use for more than 1 year.

The preferred CSPs for chiral SFC analysis have long been the modified polysaccharide materials originally developed by Okamoto and co-workers [11], and commercialized by Daicel, Chiral Technologies (Chiralpak AD, Chiralcel OD, *etc.*). In recent years some of these materials have become free of patent protection, and a number of vendors have begun to offer similar products, reputed to have comparable or even improved performance. In addition, a number of completely new products based on the modified polysaccharide motif have been introduced. In this study, we examined six of these new product introductions, including two generic versions of the Chiralcel OD-H CSPs (RegisCell and Kromasil 3-Cellucoat) and four fundamentally new CSP introductions Chiralpak IC, Sepapak-2, Sepapak-3 and Sepapak-4.

We have previously reported the use of a standardized approach for chiral SFC method development screening [12]. This approach has proven useful for the enantioseparation of many types of molecules, and the methods have been adjusted and optimized over the years to maximize probability of success. Interestingly, new CSPs being evaluated using these same conditions might be at somewhat of a disadvantage if their optimal operating conditions differ significantly from those of the 'standard' CSPs. Consequently, some flexibility in operating conditions may be justified when evaluating new CSPs, especially when they differ substantially from the preferred materials. In the present study, the six new CSPs all belong to the same class of modified polysaccharide stationary phases as the two preferred CSPs (Chiralpak AD-H and Chiralcel OD-H) used for comparison. Consequently, a screening method very close to the standardized method was chosen. Using this standard gradient, the 48 compounds in the racemate library were screened overnight on each column. With this approach a total of about 13 h was required to evaluate each column. Besides the newly commercialized CSPs, Chiralpak AD-H and Chiralcel OD-H were also included in the study for comparison.

With the results of the initial screens in hand, we set out to compare the results from the newly introduced CSPs with those of standard columns. Fig. 2 shows separations of the enantiomers of flavanone, just one of the members of the test plates, on Chiralpak AD-H, Chiralcel OD-H, and the six new CSPs. Of the generic versions of Chiracel OD-H, RegisCell shows the best resolution with performance marginally better than the Chiracel OD-H. Of the remaining CSPs Chiralpak IC and Sepapak-3 show marginal separation whereas Chiralpak AD-H, Sepapak-2 and Sepapak-4 showed no selectivity.

An overall view of the enantioseparation results for all of the 48 compounds in the library on the 8 CSPs is shown in Fig. 3. In the graph in Fig. 3, a dark grey vertical bar over the compound number indicates a baseline enantioseparation of the compound ($Rs > 1.5$), while a light grey vertical bar represents a partial enantioseparation ($0.3 < Rs < 1.5$). The white vertical bar means that no enantioseparation of the corresponding compounds was observed owing to either no separation or no elution. Comparison of Chiralcel OD-H and the two similar 'generic' version of this CSP show all columns performing similarly, with the RegisCell product having the most baseline separations, followed by Chiralcel OD-H, Sepapak-4 and Cellucoat. Of course, some care should be taken in drawing general conclusions from these results – for example, a stationary phase with very low retention but excellent enantioselectivity for a given analyte might show partial resolution, whereas another stationary phase with greater retention and poorer selectivity might show baseline resolution. One might mistakenly conclude an improved performance for the latter CSP, when a simple change in mobile phase strength would clearly show the superiority of the former CSP. Nevertheless, a general accounting of the various compounds as either baseline, partial or not resolved, is a reasonable starting point for comparison.

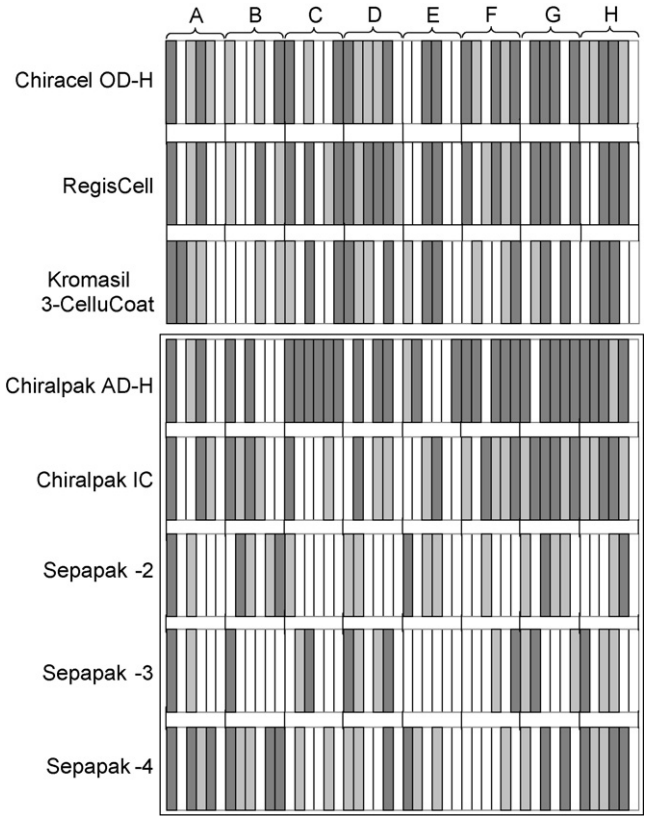

Fig. 3. Resolution maps for separation of the enantiomers of the components of the 48-member racemate library on the 8 CSPs evaluated in this study.

The selectivity maps show that the new types of CSPs (Chiralpak IC, Sepapak-2 and Sepapak-3) performed reasonably well, with some notable separations being obtained with the Chiralpak IC. A closer view indicates that Sepapak-2 and Sepapak-3 showed the worst overall performance in terms of the number of baseline and partial enantioseparations observed for the 48 library compounds.

The resolution maps shown in Fig. 3 are useful for gaining a general sense of which new CSPs perform best, however when considering which new CSP should be added into an existing general screening workflow, other considerations are equally important. For example, CSPs included in screening not only are required to provide enantioselectivity for a wide range of compounds, but also should offer orthogonal selectivities to the other CSPs in the screening system. Similarly, a new CSP that showed broad generality might seem to be an ideal addition to a screening set. However, if the CSP always delivers a resolution that is inferior to that provided by one of the other CSPs in the screening set, it would be of little value. Thus, some metric that accounts for 'best resolution' or unique solutions to a problem is also useful.

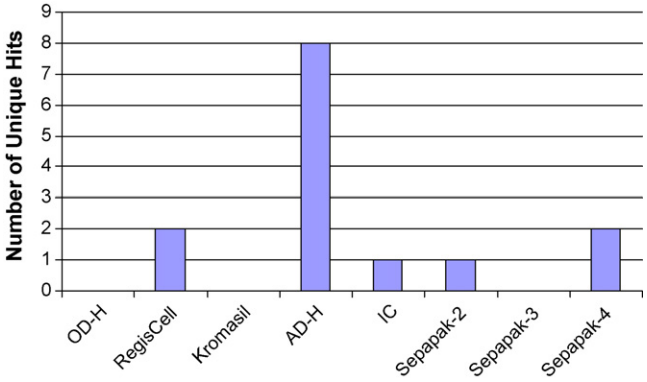

Fig. 5. Unique hits in enantioselectivity for the 8 CSPs evaluated.

With these considerations, two bar charts were constructed using the SFC screening results on the racemic compound library to show the baseline hits for the 8 CSPs evaluated, as shown in Fig. 4. Of the generic versions of the Chiralpak OD-H column (Fig. 4a), RegisCell showed the best results, followed by Chiralcel OD-H and Kromasil Cellucoat. Of the other fundamentally new CSPs (Fig. 4b), Chiralpak IC showed the best performance, although not comparable to Chiralpak AD-H. When all eight CSPS are shown together, Chiralpak AD-H can be seen to provide the best overall results. Consequently, a researcher allowed to choose only one CSP would do well to choose Chiralpak AD-H. If permitted additional choices, RegisCell followed by Chiralpak IC, Sepapak-4, OD-H, Cellucoat and Sepapak-2 would be advisable.

Among the new CSPs, RegisCell performed by far the best, with 14 best separations and 1 unique separation. Chiralpak IC and Sepapak-4 also gave good performance with 4 best separations for each, and 2 unique separations for Sepapak-4 and 1 for Chiralpak IC. Sepapak-2 gave 1 best and 1 unique separations. Cellucoat gave only 1 best separation, and Sepapak-3 gave no best or unique separation in our study. Based on these results, RegisCell, Chiralpak IC, and Sepapak-4 were incorporated into standard SFC screening systems in these laboratories. In general, it is valuable to not only identify the good columns but also to quickly identify poorly performing CSPs at an early stage.

The number of unique hits for each of the 8 CSPs is illustrated in Fig. 5, again showing that the AD-H CSP gave both the greatest number of best separations and the greatest number of unique separations among all 8 CSPs.

The direct comparison of two different CSPs is often of value. From the colored selectivity maps shown in Fig. 3, RegisCell, a generic Chiralcel OD-H column seemed to give comparable performance to the original Chiralcel OD-H, while Figs. 4 and 5 shows

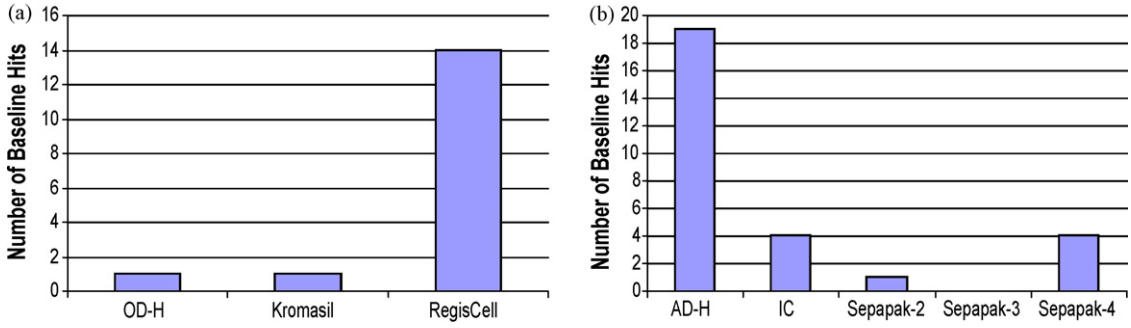

Fig. 4. Baseline hits in enantioselectivity for the 8 CSPs (a) Chiralcel OD-H like CSPs (b) Chiralpak AD-H with 4 other CSPs.

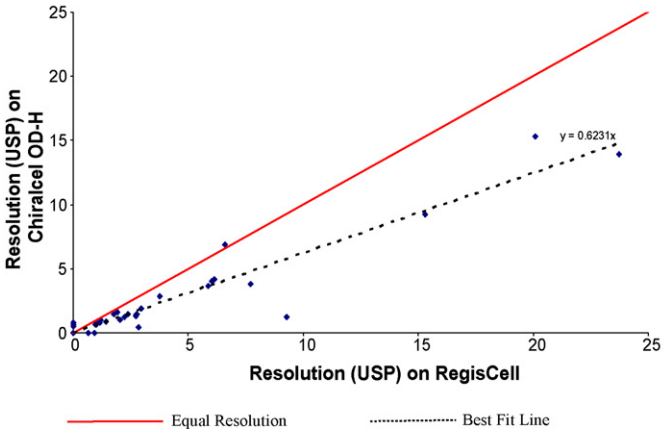

Fig. 6. Resolution map of RegisCell and Chiralcel OD-H permits direct comparison the relative ability of the two columns to resolve the enantiomer of each of the 48 members of the standard racemate library.

RegisCell often provides better separation among the OD-H CSPs evaluated. In order to better understand these differences in performance, a resolution map was constructed for the 2 CSPs using the resolution calculated from the SFC screening results for each of the 48 compounds, as shown in Fig. 6. Each data point in the graph represents a compound in the library, with the resolution on RegisCell shown on *x*-axis, and the resolution on Chiralcel OD-H on *y*-axis. If the 2 columns performed equally, the data points should fall equally on the solid red line. (For interpretation of the references to color in this sentence, the reader is referred to the web version of the article.) In our study, most of the data points fell under the equal resolution line, which means that for most compounds in the library the RegisCell CSP gave better resolution compared to the original Chiralcel OD-H CSP. It is also interesting to note that the data points loosely follow a linear correlation, perhaps relating to the fact that the 2 columns contain very similar CSPs.

The results for SFC column screening for each of the 48 compounds in the racemate library were recorded in a commercial ACD database. This database is an extension of the well-known Chirbase database [13] created by Prof. Christian Roussel and Dr Patrick Piras at Université Paul Cézanne, Marseilles. The database contains over 100,000 published and unpublished chiral chromatographic methods for 30,000 different compounds, and is searchable by all chromatographic parameters as well as by structure and structure similarity. ChirBase for ACD/Labs allows the user to maintain an 'in-house' database of separation methods using the Chirbase format. This tool is understandably of considerable value in centers where large numbers of enantioseparation methods are developed. Not only is the database useful for archiving purposes, but also a quick search of existing methods can often be used to identify previously developed methods for known compounds. In addition, this general approach could be of value in gaining a better understanding concerning the mechanism of enantioseparation of different compounds on different types of CSPs.

4. Conclusion

A streamlined procedure was established for systematic evaluation of new CSPs for SFC enantioseparations. A racemic compound library consisting of structurally diverse commercial and proprietary drug compounds was prepared in 96-well microplates to allow convenient storage and transfer. By using standardized SFC methods, a new CSP can be fully evaluated with an overnight run. The SFC results of enantioseparations of the 48 library compounds on a new CSP can be compared to those from the existing CSPs or other new CSPs. The comparison identifies a column that provides enantioseparation of a diverse library of racemates but also identifies which CSPs afford the best and/or greatest number of unique separations which is an important factor when selecting the most effective CSPs for an inclusion in a chiral screening system. In addition, direct comparison of 2 columns using a resolution map constructed using SFC evaluation results also helps to understand the underlying performance differences between the columns. The systematic evaluation approach not only applies to the SFC chiral columns, but also can be used in reversed phased or normal phase HPLC mode. With an increasing number of new CSP product introductions each year, streamlined approaches will help to quickly identify the most useful columns for use in the pharmaceutical research environment.

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