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Rapid method development for chiral separation in drug discovery using multi-column parallel screening and circular dichroism signal pooling

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Abstract

A novel strategy for rapid chiral method development has been developed using multi-column parallel screening and circular dichroism (CD) signal pooling. Described is the first use of a customized HPLC system that integrates an HPLC auto-sampler, one pump and five divided channels with five columns and five UV detectors to screen five chiral stationary phases (CSPs) simultaneously in parallel. A high-pressure semi-prep on-line pre-filter, a six-port manifold and five individually adjusted backpressure restrictors were installed in the system which allowed the sample and mobile phase to be evenly distributed over the five columns and UV detectors. The five CSPs, namely Chiralpak AD and AS, Chiralcel OJ and OD and Whelk-O1, were screened. The system guarantees a five-fold increase in speed for chiral column scouting compared with the widely used automated sequential column switching approach, and does not have the limitations of the coupled column screening approach for enantiomers whose elution order could be reversed on CSPs. Furthermore, the five channels after the UV detectors were recombined using a reversed flow splitter into a CD detector. The pooled CD signal from the five channels was recorded to track the elution order of the resolved enantiomers and to determine their sign, positive or negative. The signal pooling allows for the effective use of a single CD detector for multiple columns since unresolved racemate has little CD signal, and observing the sign of CD signal for one of the two enantiomer UV peaks is sufficient for tracking the enantiomeric elution order.

Keywords: Chiral chromatography; Enantiomeric separation; Circular dichroism detector; Chiral column screening

1. Introduction

There is a broad range of examples where the stereoisomers of therapeutic drugs show significant differences in their pharmacological, pharmacokinetic or toxicological effects in biological systems [1,2]. In addition, the US Food and Drug Administration (FDA) requires the evaluation of the safety and effectiveness of each individual enantiomer, as well as their combination, if the new drug contains asymmetric centers [3]. Therefore, chiral separations have become an important part of drug discovery and development, and the majority of today's top-selling 500 drugs are single enantiomers [4]. In the early stages of drug discovery, time constraints are often crucial to achieve chiral resolution and provide small amounts (50 mg −10 g) of both pure enantiomers for preliminary comparative biological testing. As chiral chromatographic separation usually furnishes both enantiomers in high optical purity, chiral HPLC has become one of the most utilized techniques to separate and isolate pure enantiomers in drug discovery [5]. Consequently, faster chiral HPLC method development continues to be an area of tremendous research interest. Recently, we have published a "Simulated Moving Columns" technique to achieve baseline enantiomeric resolution on chiral columns that have insufficient chiral resolution power [6]. The work described herein is a continue effort to search for new strategies for accelerating chiral HPLC method development.

There are 1300 chiral stationary phases (CSPs) and approximately 170 of them are commercially available today [7]. Although almost any enantioseparation can be achieved with at least one of these chiral selectors using

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well-established chromatographic techniques (HPLC, SFC, CE and GC), only for a limited number of selector-selectant combinations chiral recognition modes have been developed [8]. Reliable and rapid enantioselectivity prediction of a new chiral molecule with almost any CSP is not currently available. Small changes in structure and/or chromatographic environment have shown great impact on the ability to resolve a racemate for many CSPs, especially the popular polysaccharide derivatives types of CSPs. Therefore, "trial-and-error" screening of a set of CSPs that offers a broad-spectrum of enantioselectivity has been the usual approach to find the best chiral selector for developing enantiomeric separations in the pharmaceutical industry.

Currently, there are two automated column screening approaches, automated column switching in sequence and screening using column coupling. Automated column and/or solvent screening in sequence using column switching has been reported several times in the past [9,10] and is the most widely used approach for chiral method development today. However, since the columns are tested one at a time under each mobile phase condition, this approach often takes more than 1 day for four or more columns and mobile phases. The coupled column screening approach was proposed by Wang et al. [11] using macrocyclic glycopeptide columns. Three different 100 × 4.6 mm columns were connected in series and screened as one column using three different mobile phases. Since three CSPs are screened simultaneously, it is very rapid. However, this approach cannot be used for many other CSPs such as polysaccharide-derivatives CSPs, since the elution order of an enantiomeric pair can often reverse between different columns [12,13]. Even on macrocyclic glycopeptide columns elution order reversal has been observed. For example, elution order is reversed for 4-benzyl-2-oxazolidinone on CHIROBIOTIC R and CHIROBIOTIC V columns (ASTEC, Whippany, NJ, USA) using 50/50 *n*-hexane/ethanol [14]. Moreover, screening using column coupling does not identify the best column to use. Deconvolution to determine the optimum CSP by screening each of the coupled columns individually has to be done using the selected mobile phase system. This increases the screening time and makes it more difficult to automate the entire column selection process.

A novel strategy and a customized HPLC system were developed and are described herein for rapid chiral column scouting using multi-column parallel screening. The modified HPLC system allows us to simultaneously screen five CSPs in parallel using a regular HPLC auto-sampler and a pump with five UV detectors. A two-column parallel HPLC with a single multi-wavelength absorbance detector was reported [15] to increase chemical selectivity by splitting a sample onto two parallel columns and then recombining them into a single multi-wavelength absorbance detector. Chemometric data analysis with the generalized rank annihilation method (GRAM) was used to identify and regain the resolution for quantification of the analytes. However, the multivariate data analysis methods based on UV spectra are not applicable for enantiomeric separation using CSPs since the enantiomer pair has identical UV spectrum. A four-column parallel chromatography system for LC/MS analyses was also reported [16] and a four-channel Multiplex LC/UV/MS (MUX) System is commercially available from Waters Corp. (Milford, MA, USA). These systems were designed for high-throughput parallel LC/MS analyses using staggered injections on four identical columns with a single mass spectrometry. Although we demonstrated in this study that MUX system after several modifications may be used to screen chiral columns, the purpose of this work was to develop a more cost effective HPLC system specifically designed for the purpose of parallel chiral column screening.

Since the enantioselectivities of most CSPs are unpredictable, keeping track of the elution order of the resolved enantiomers is important, not only to understand the chiral recognition mechanism, but also for trace level detection in highly enriched enantiomers and for scaleup to chiral preps where elution order has great impact. An on-line LC–circular dichroism (CD) detector facilitates peak tracking as well as the determination of the sign of chirality of the resolved enantiomers with high sensitivity during the chromatographic separation [17]. However, LC/CD detectors are relatively new and not inexpensive. Thus, in order to use a single CD detector for the parallel column screening, a CD signal pooling technique was used. Flow from the five UV detectors was combined and pumped through one CD detector, and the pooled CD signal was used to determine the sign of the chirality of the enantiomers separated on one or more of the five columns.

2. Experimental

2.1. Chemicals

The commercially available chemical, trans-stilbene oxide and Sulconazole were purchased from Sigma–Aldrich (St. Louis, MO, USA), and Thalidomide was purchased from ICN Biomedicals, Inc. (Aurora, Ohio) The Wyeth discovery compounds used in the study are small drug molecules (MW, 250–500) and were synthesized in-house. Methanol (MeOH), ethanol (EtOH), 2-propanol (IPA) and *n*-hexane were HPLC-grade solvents purchased from Mallinckrodt Baker (Muskegon, MI).

2.2. Chiral stationary phases

Chiralpak AD and AS (amylose derivatives), and Chiralcel OD and OJ (cellulose derivatives) were purchased from Chiral Technologies (Exton, PA). The brush-type column, Whelk-O1, was purchased from Regis Technologies, Inc. (Morton Grove, IL).

2.3. Instrumentation

An Agilent 1100 HPLC System (Agilent Techologies, Wilmington, DE, USA) was used which included G1313A

auto-sampler, G1313quat pump, G1322A vacuum degasser and a G1315A diode array UV detector. Four additional used UV detectors, one Agilent 1050 variable wavelength detector and three Waters 486 tunable absorbance detectors (Waters Corp., Milford, MA, USA), were used to configure the parallel column screening system. The CD detector, Jasco CD1595, was purchased from Jasco International Co., Ltd. (Maryland, USA). Two Agilent 35900E Interfaces (A/D converters) were used to acquire the UV and CD signals from the additional UV and CD detectors for processing by Agilent's ChemStation® chromatography data system. A high-pressure semi-prep inline filter with a 2-μm stainless steel frit of 0.625 diameter from Upchurch Scientific (Pompton Plains, NJ, USA) was used for pre-column filtering and mixing. The PEEK six-port manifolds with 1/16 in. fittings used as flow splitter and reverse flow splitter were also from Upchurch, as well as the PEEK tubing (0.005 and 0.007 in. i.d.) used for pressure adjustment.

3. Results and discussion

Enantioselectivities of most CSPs are unpredictable for a particular enantiomeric pair, and it is commonly observed that small changes in structure and/or chromatographic environment can greatly affect the ability to resolve a racemate for a given CSP. As an example, different CSPs were tested for chiral resolution of two isomers, A and B (Wyeth discovery compounds), whose planar structures differ only by the substitution pattern of Br, F and OH on their structure core (see Fig. 1). For isomer A, the enantiomeric pair was resolved on the Whelk-O-1 and Chiralcel OD columns, but not on the Chiralpak AD column. However, the elution order on the Whelk-O-1 and Chiralcel OD columns was reversed as evidenced by the positive and negative CD signals (see the LC/CD chromatograms on top). For isomer B, the enantiomeric pair was not resolved on the Whelk-O-1 column but rather on the Chiralcel OJ and Chiralpak AD columns. The elution order on the OJ and AD columns was again reversed. In addition to the CSPs and small structure changes, slight change in mobile phase composition can also affect the elution order, exemplified by the chiral separation of a Wyeth discovery drug molecule C (enriched) shown in Fig. 2. The reversal of elution order phenomena have also been reported for some commercially available small molecules on Pirkle-type [18] and polysaccharide CPSs [12,13,19,20].

Due to the complex and unpredictable nature of enantiomeric HPLC separations, chiral method development requires empirical and "trial-and-error" approaches for selecting a suitable CSP and mobile phase. HPLC systems with automated column switching in sequence have been widely used to facilitate this tedious selection procedure for

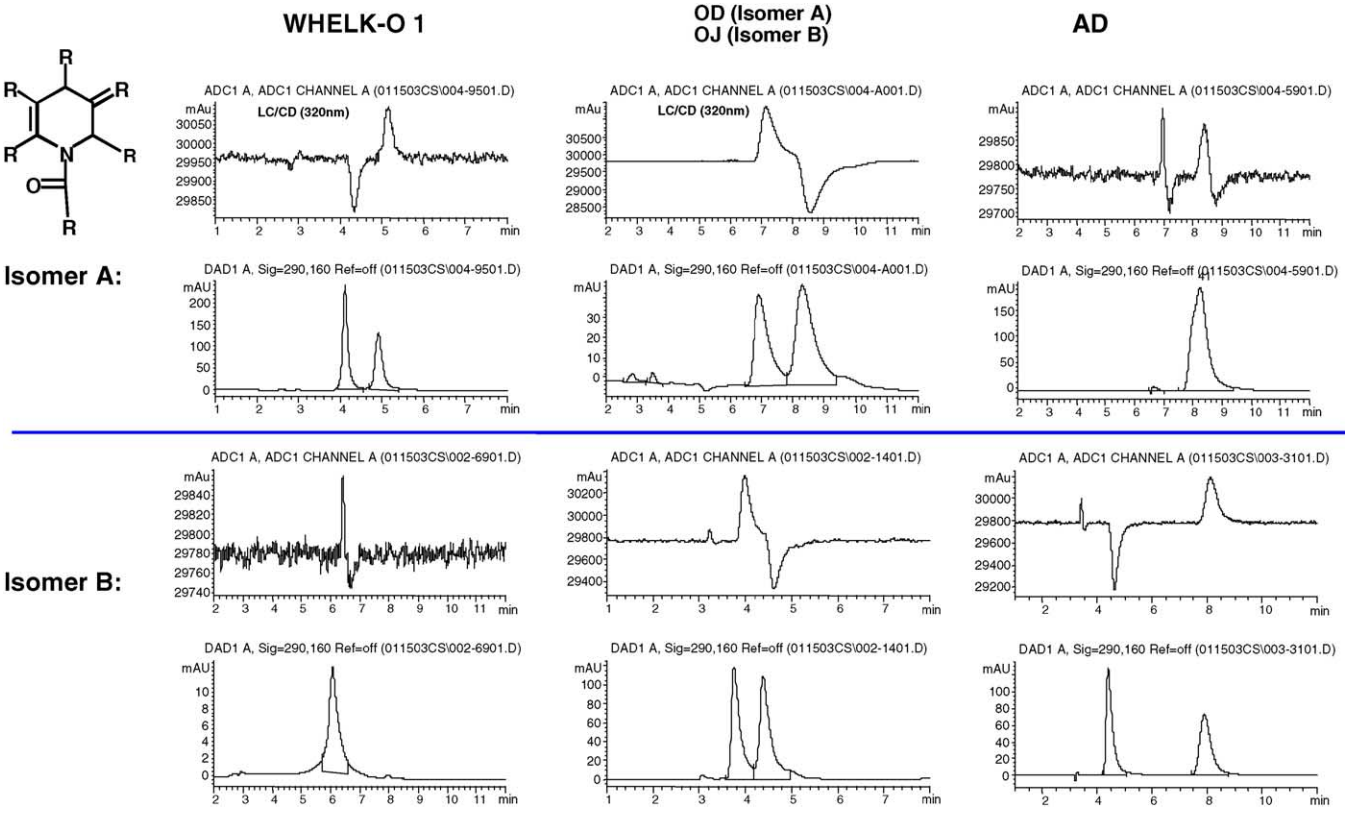

Fig. 1. Chiral column screening results of Wyeth discovery compounds (isomers A and B) whose planar structures were different by the substitution pattern of Br, F and OH to their structure core). The LC/CD chromatograms (at 320 nm) on the top and LC/UV chromatograms (at 220–370 nm UV window) on the bottom for each isomer show the resolution and elution order reversal between different CSPs. Mobile phase: 10% ethanol in *n*-hexane; flow rate: 1 mL/min.

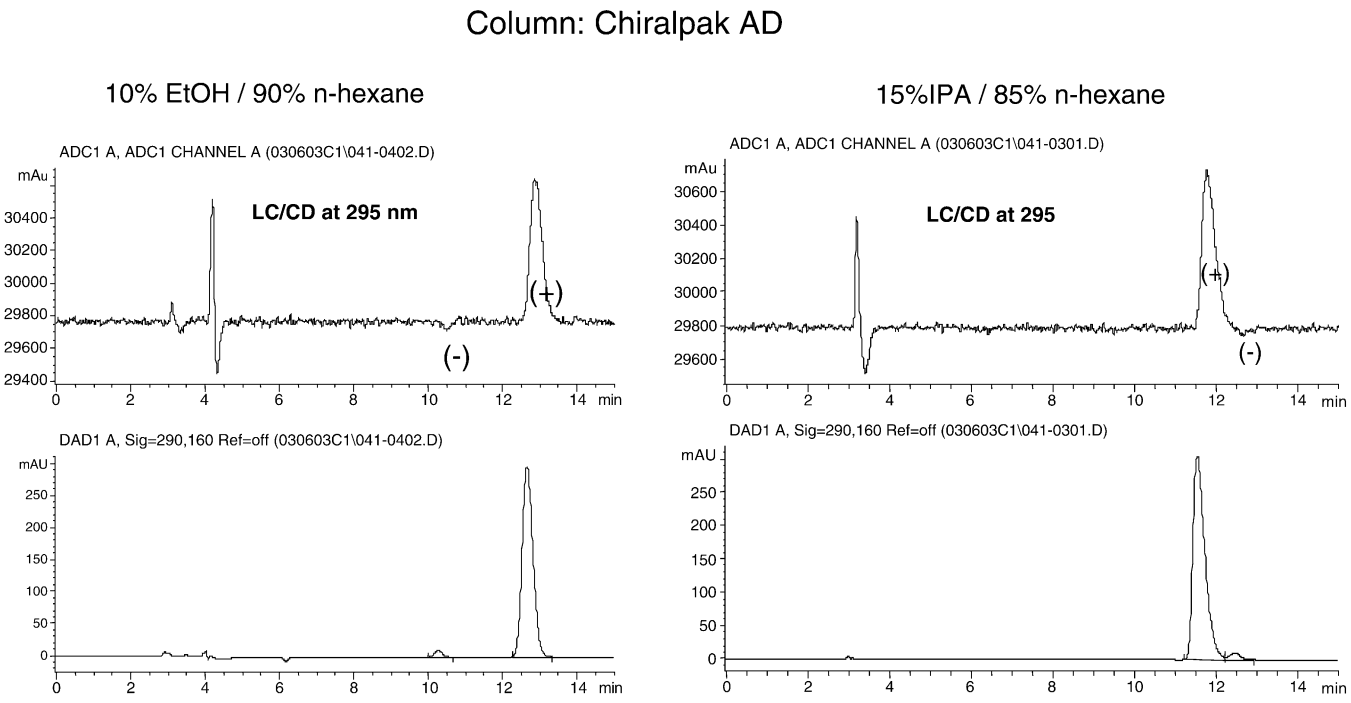

Fig. 2. Elution order reversal on Chiralcel AD column due to the change in mobile phase for Wyeth discovery compound C (enriched). On the top is the LC/CD chromatogram at 295 nm and on the bottom is the LC/UV profile at 220–370 nm. Mobile phase as indicated; flow rate: 1 mL/min.

chiral method development. However, testing of multiple columns one at a time with various mobile phases multiplies quickly with respect to time. A new strategy for CSP screening, namely multi-column parallel screening, was developed to dramatically increase the speed of this chiral column selection process. Rather than screening chiral columns in series by column switching, five columns were screened simultaneously in parallel. To our knowledge, no commercial HPLC system has been designed to perform such a task to date. The closest system that could be used for this purpose is the four-channel Multiplex LC/UV/MS (MUX) System from Waters Corp. (Milford, MA, USA) that was designed for high-throughput parallel LC/MS analyses using four identical columns. To prove initially the concept of parallel chiral column screening, we modified the MUX system illustrated in Fig. 3 to screen Chiralpak AD and AS and Chiralcel OD and OJ columns. The system was equipped with a Waters 1525 binary pump that delivered the mobile phase to a Leap HTC PAL injector with four separate injector seats and loops. A sample was injected once into each of the

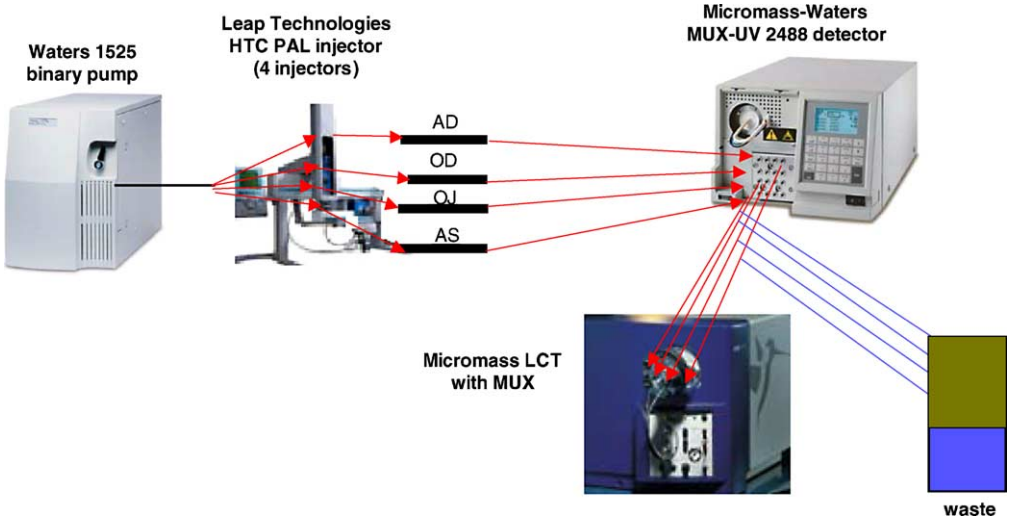

Fig. 3. A modified four-channel Multiplex LC/UV/MS (MUX) System. The four flow paths out of the MUX 2488 UV detector were connected to pressure restrictors, and then directed to waste bypassing the Micromass LCT.

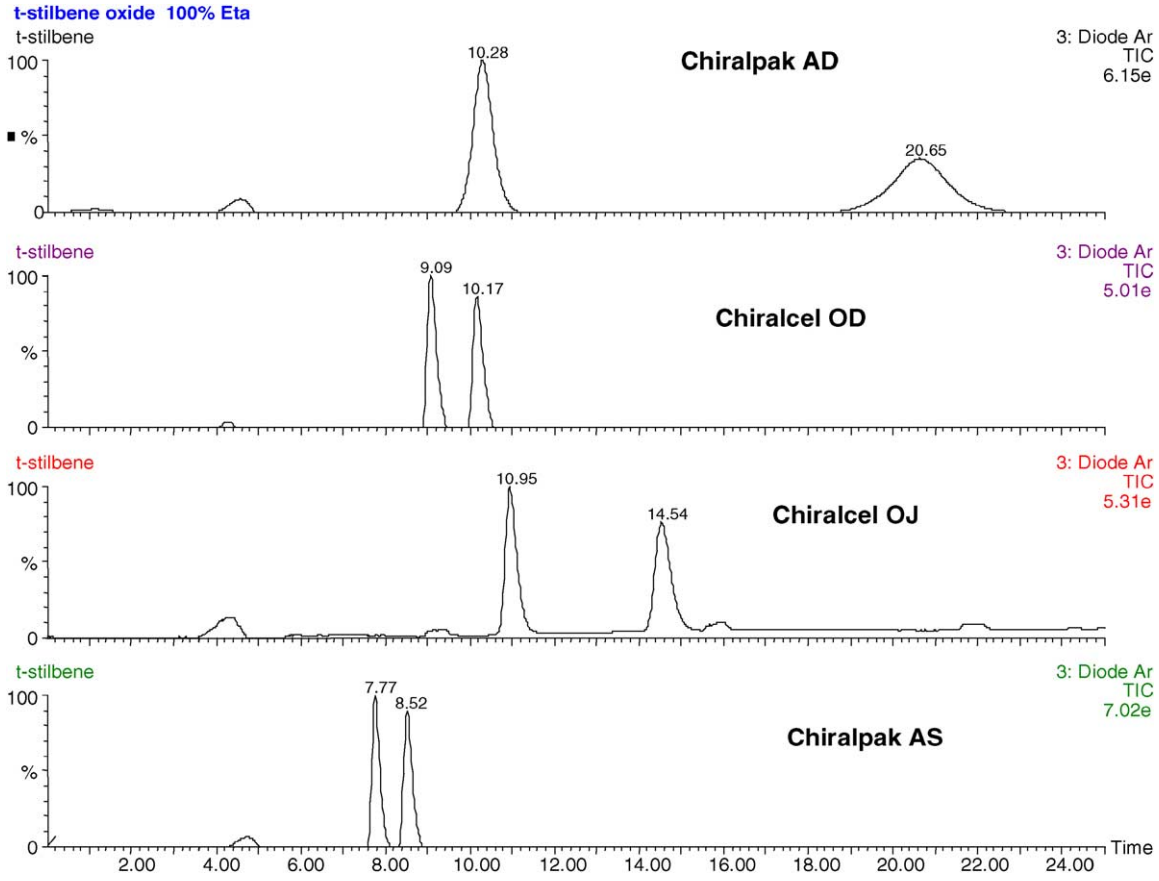

Fig. 4. Parallel chiral column screening chromatograms of trans-stilbene oxide using the modified MUX system. Mobile phase: 100% ethanol; flow rate: 0.5 mL/min for each column. UV wavelength: 210–360 nm.

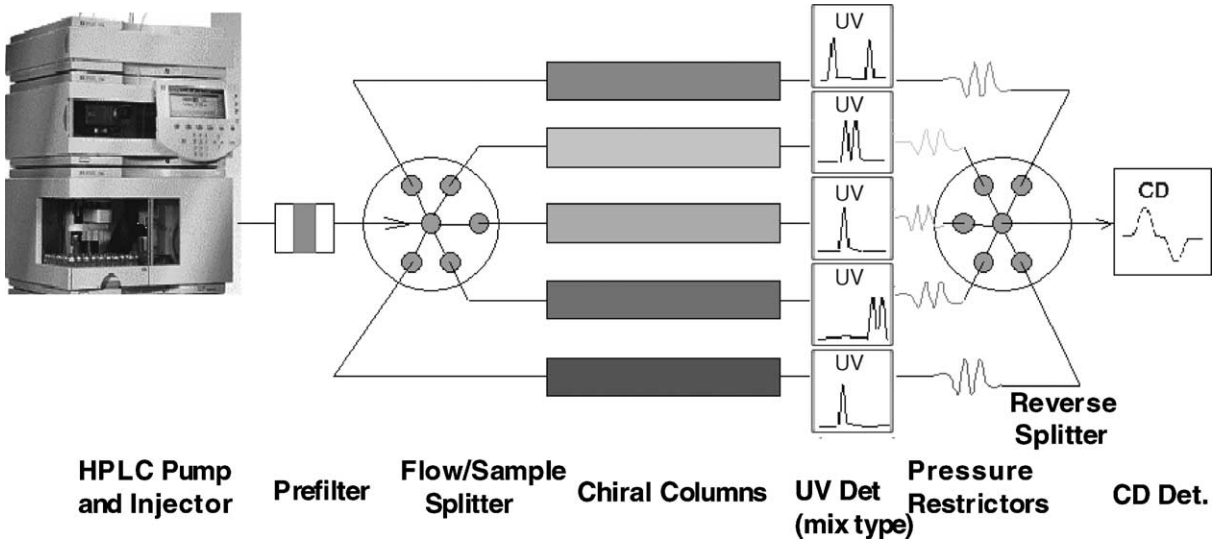

Fig. 5. Customized HPLC system for five-column parallel screening with CD signal pooling.

four loops then carried to four columns simultaneously. A MUX-UV 2488 detector was used to acquire the UV signal. A Micromass LCT with MUX for high resolution MS detection was taken out of the system, as it was not needed for our purpose. Since a single pump was used to divide and deliver the mobile phase flow into four channels, a difference in column backpressure on the system would result in different flow rates between the columns. To solve this problem, we used the PEEK tubing added onto the outlet of each channel from the UV detector to serve as pressure restrictors by carefully adjusting their diameters and lengths to equalize the backpressure in each channel. Parallel column testing using trans-stilbene oxide was achieved on the modified MUX system as shown in the parallel chromatograms in Fig. 4. The components on the MUX system, particularly the MUX multi-channel UV2488 detector, can not be used separately since they can only be controlled and communicated by the MUX Masslynx software. In addition, four separate injectors were used for each column, any particles or trace precipitates from the sample could accumulate and result in partial clogging of one or more channels, destroying the pressure balance between the columns and leading to significant flow rate difference between the columns.

To develop an inexpensive HPLC system for multi-column parallel screening, an Agilent 1100 HPLC system with four additional (old and used) UV detectors was configured as shown in Fig. 5. The outlet of the auto-sampler was connected to a high-pressure on-line semi-prep pre-filter (2 μm). The purpose of the pre-filter was two-fold. First, it stopped any particles entering into the divided flows that could cause clogging and leading to the flow imbalance between the channels. Second, it also functioned as a quick mixer of the sample with the mobile phase prior to its dividing into separate columns. A flow splitter (six-port manifold) was used to divide the flow along with the sample evenly into five columns, and subsequently to the five UV detectors. The even flow distribution was regulated by the equal backpressure in each channel precisely adjusted using PEEK tubing with different diameters and lengths. The tubing was installed after the UV detectors to prevent any additional band broadening in the separation. The backpressure adjustments were performed using 50:50 *n*-hexane/ethanol, and the pressure in each channel was equalized to within 0.5 bar. The flow rate in each channel was then measured using 50/50 *n*-hexane/ethanol, 90/10 *n*-hexane/2-propanol and 100% ethanol separately. To obtain 1 mL/min flow through each column, a 5 mL/min flow rate was set for the pump. Less than 2% variation in flow rates between the five channels was observed. The five columns installed on the system are Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H and Whelk-O1. The chromatograms from the parallel chiral column testing using trans-stibene oxide are shown in the top five LC/UV chromatograms in Fig. 6. Such a test

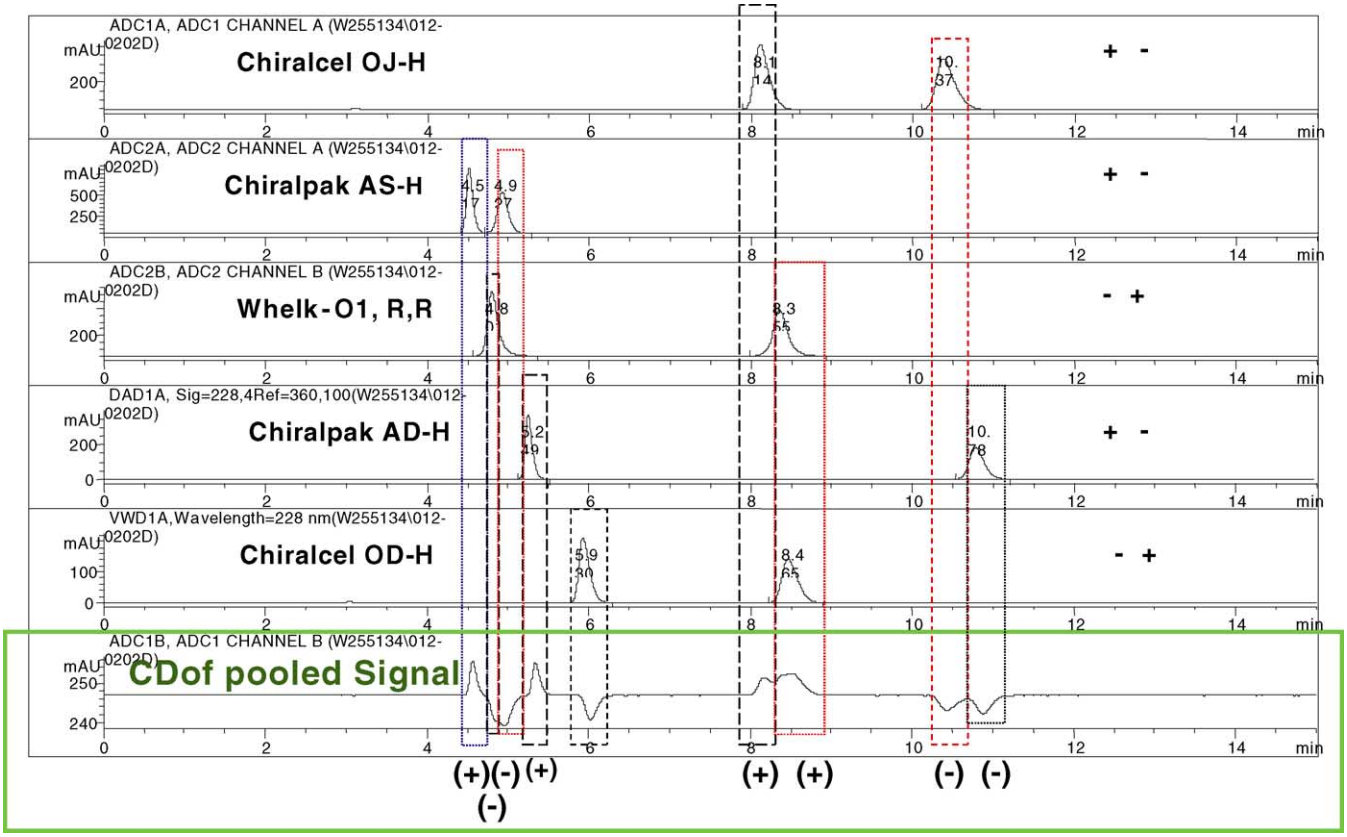

Fig. 6. Parallel chiral column screening chromatograms of trans-stilbene oxide using the customized HPLC system. Mobile phase: 10% 2-propanol in *n*-hexane; flow rate: 1 mL/min for each column. UV wavelength: 225 nm, CD wavelength 240 nm.

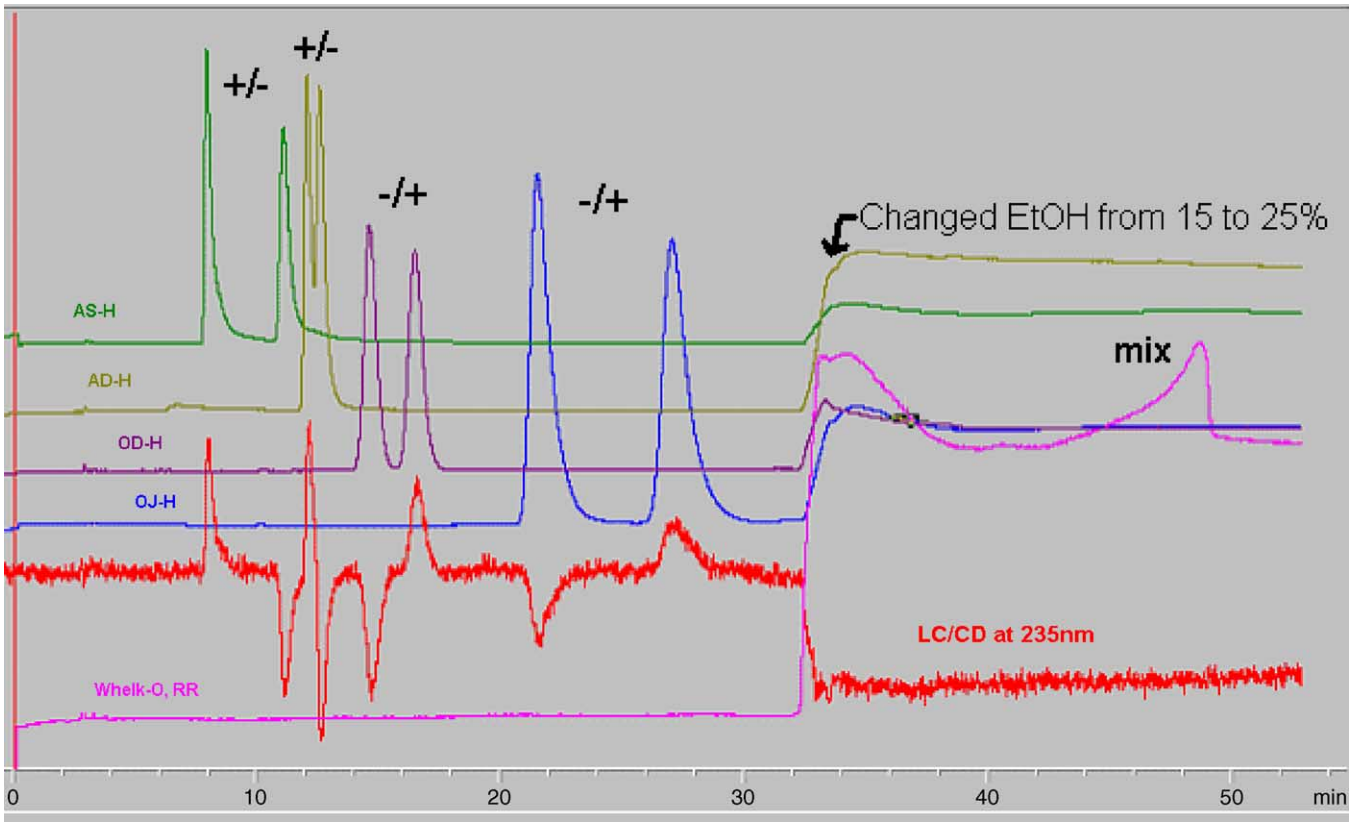

Fig. 7. Parallel chiral column screening chromatograms of Sulconazole with CD signal pooling. Mobile phase: 15% ethanol in *n*-hexane from 0 to 30 min and 25% ethanol in *n*-hexane from 30 to 55 min. Flow rate: 1 mL/min for each column. UV wavelength: 225 nm, CD wavelength 235 nm.

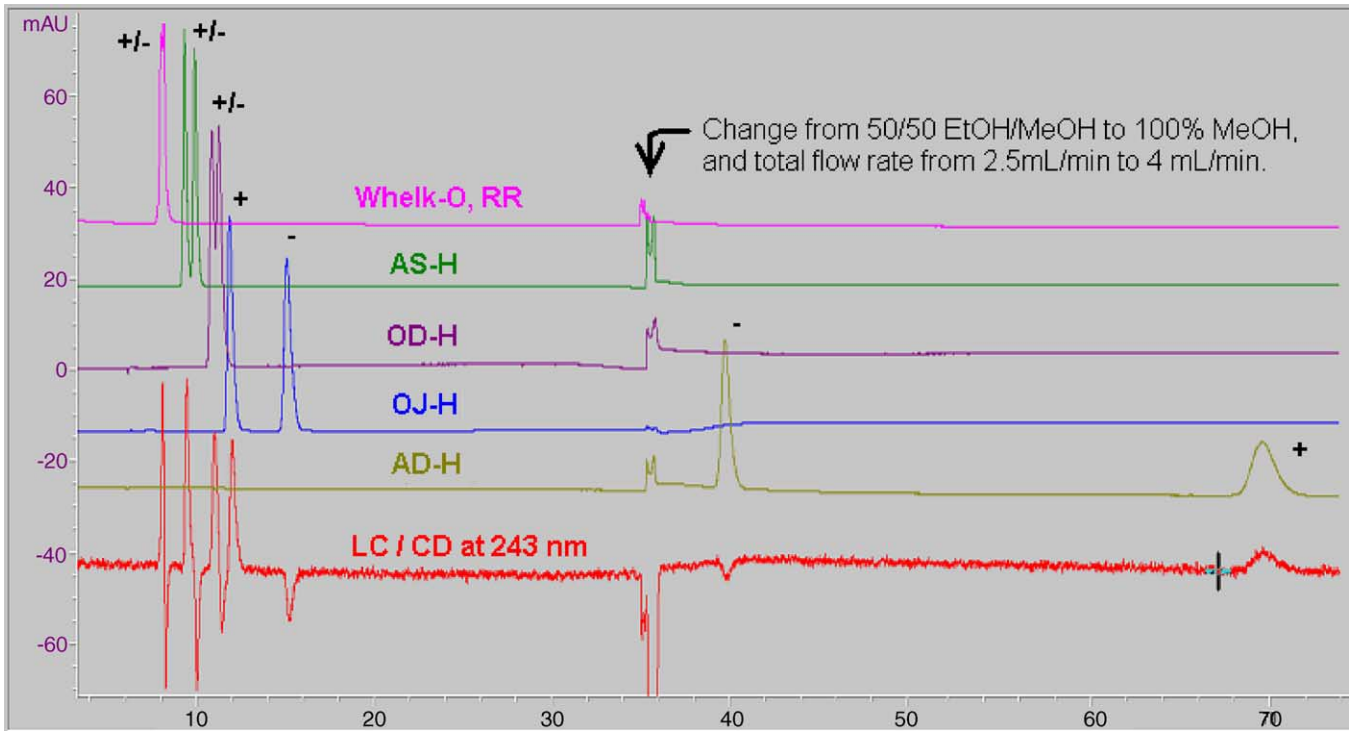

Fig. 8. Parallel chiral column screening chromatograms of Thalidomide with CD signal pooling. Mobile phase: 50/50 ethanol/methanol from 0 to 30 min and 100% methanol from 30 to 75 min. Flow rate: 0.5 mL/min for each column from 0 to 30 min and 0.8 mL/min from 30 to 75 min for each column. UV wavelength: 225 nm, CD wavelength 235 nm.

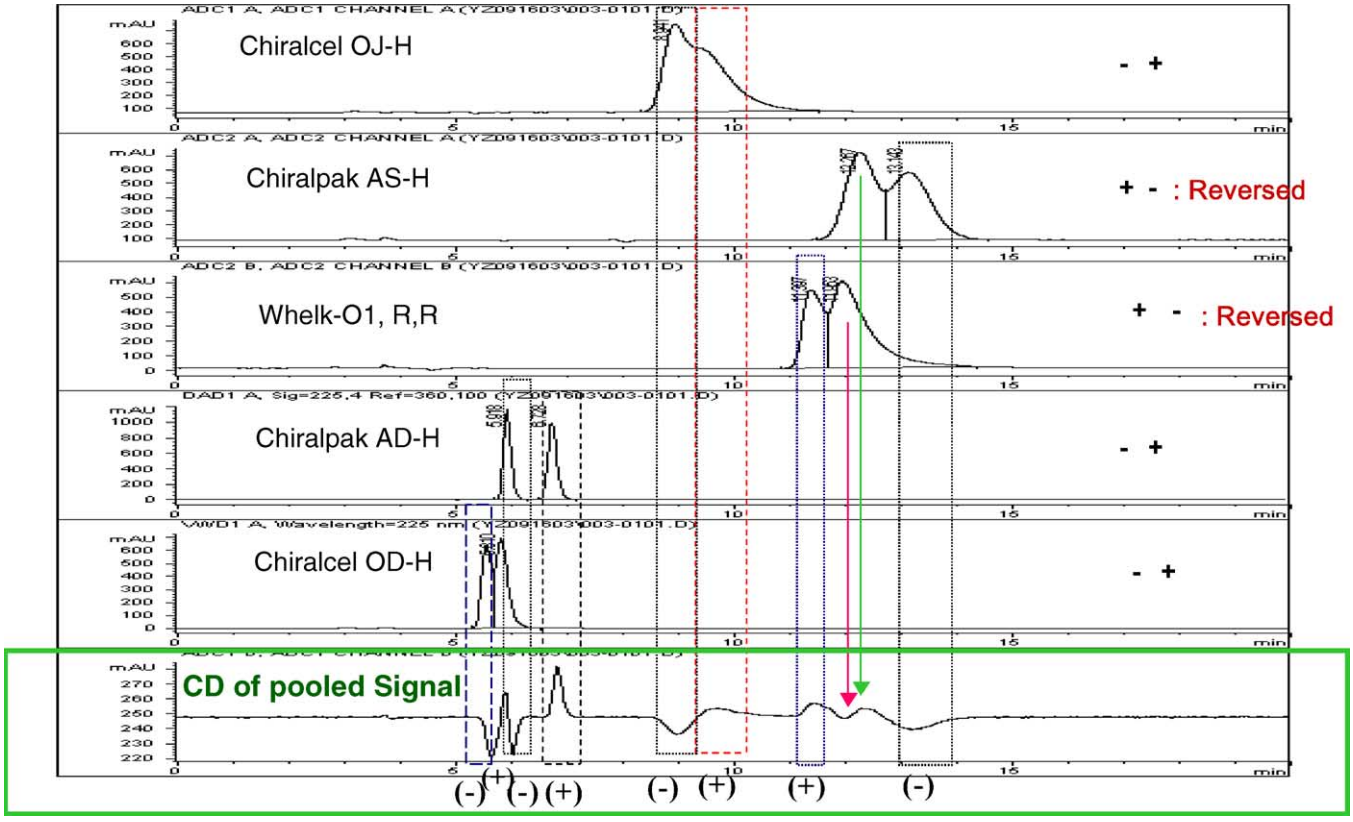

Fig. 9. Parallel chiral column screening chromatograms with CD signal pooling for Wyeth discovery compound D. The top five are the LC/UV trace (at 225 nm) on each CSPs. The bottom is the pooled LC/CD signal (at 265 nm) which revealed the + and − sign of the resolved enantiomers as indicated below the chromatogram. The resulting elution order is indicated in each LC/UV chromatogram.

that normally required five injections and 75 min now took only 15 min with a single injection using the parallel column system.

On-line monitoring of CD signals during a chiral HPLC separation is a powerful detection technique for chiral chromatography. An LC/CD signal can reveal information about the sign of chirality of resolved enantiomer peaks. During a chiral column screening, a CD detector helps in tracking the elution order based on the chirality. In addition, since an achiral molecule does not yield a CD signal, a CD detector can be used to distinguish enantiomers from achiral impurities. However, because CD detectors are not inexpensive, the possibility of using one CD detector for more than one chiral column was pursued by developing a CD signal pooling technique. As shown in Fig. 5, the five channels after the UV detectors were recombined using a reversed flow splitter into a high-pressure flow cell of a CD detector. At an appropriate wavelength, a CD detector always yields a positive and a negative peak for a pair of resolved enantiomers, therefore, the positive or negative sign of either one of the two enantiomer peaks is sufficient to reveal the chirality of both. When pooling the LC/CD signals from all five channels, some components from different columns may co-elute. However, since there is no or little CD signal from chiral columns that do not resolve the racemate, coelution of an enantiomer with an unresolved racemate has no adverse impact on the CD detection. The chance that one of the two separated enantiomeric peaks remains separated after pooling is much greater than the chances that all resolved enantiomers are coeluted. The multi-column parallel screening with CD signal pooling has been used routinely in our laboratory for the past 6 months, and rarely has there been a case where all resolved enantiomers coeluted during signal pooling. This setup has proven to be more effective than alternatively installing the CD detector after only one of the five UV detectors. The pooled CD signals for trans-stilbene oxide are shown in the bottom LC/CD chromatogram of Fig. 6, and the vertical dotted boxes outline the correlation of the CD signals to the LC/UV peaks. The CD signal pooling technique allowed the peak tracking for all five columns and clearly revealed that the elution order of the enantiomers on the OJ-H, AS-H and AD-H columns were reversed on the OD-H and Whelk-O1 columns.

Two commercially available drug molecules, Sulconazole and Thalidomide, were used to demonstrate the technique. As shown in Fig. 7, the positive and negative LC/CD peaks clearly reveal the elution order reversal for the Sulconazole enantiomers between Chiralpak AD-H and AS-H columns and Chiralcel OJ-H and OD-H columns using 15% ethanol in *n*-hexane. Ethanol was increased to 25% at 30 min to elute the unresolved Sulconazole out of the Whelk-O1 column. Fig. 8 shows the parallel column screen chromatograms for Thalidomideusing 50/50 ethanol/methanol, and the sign of

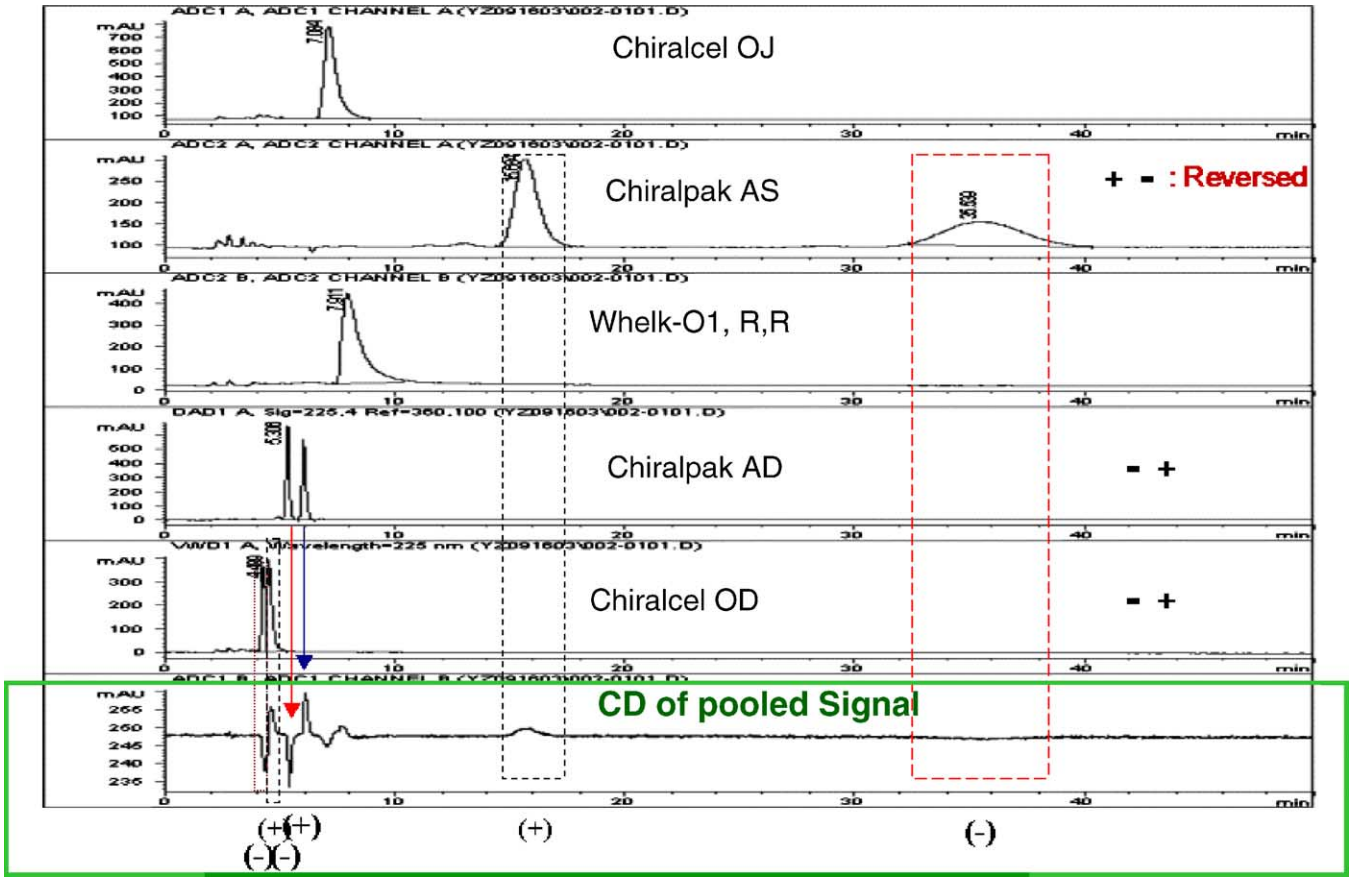

Fig. 10. Parallel chiral column screening chromatograms with CD signal pooling for Wyeth discovery compound E. The top five are the LC/UV trace (at 225 nm) on each CSPs. The bottom is the pooled LC/CD signal (at 265 nm) which revealed the + and − sign of the resolved enantiomers as indicated below the chromatogram. The resulting elution order is indicated in each LC/UV chromatogram.

chirality of each UV peak is clearly indicated by the LC/CD signal at the bottom. At 30 min, the mobile phase was changed to 100% methanol and flow rate was increased from 2.5 to 4 mL/min to elute the enantiomers from the highly retained AD-H column in this case. Two additional examples of chiral column screening for two Wyeth discovery compounds D and E are given in Figs. 9 and 10. In Fig. 9, an enantiomeric elution order of compound D on Chiralpak AS-H and Whelk-O1 columns were reversed compared with that on the other polysaccharide-derivative CSPs. Since the enantiomers of compound E shown in Fig. 10 were not separated on the Whelk-O1 and OJ-H columns, little pooled CD signal was observed. The sign of chirality of the resolved enantiomers on the other three columns were illustrated, and elution order reversal was found between the AS-H column and the AD-H or OD-H columns.

4. Conclusion

A novel strategy for rapid chiral method development has been developed using multi-column parallel screening and CD signal pooling. The customized HPLC system described herein was inexpensive to set up since any UV detectors can be used, and demonstrated to be quite robust. The system allows five CSPs to be screened simultaneously in parallel and leads to five-fold improvement in efficiency for chiral method development. A screening of five columns and five mobile phases that would require 10 h (and usually overnight runs) could be accomplished in 2 h (assuming a 25 min run times with 4.6 mm × 25 cm columns). In addition, the CD signal pooling technique allowed us to use a single CD detector for multiple columns. The importance and usefulness of tracking resolved enantiomers and determining their chirality using the pooled CD signal for multiple columns were demonstrated on both commercially available and Wyeth discovery compounds.

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