Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 964–969

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Development of LC chiral methods for neutral pharmaceutical related compounds using reversed phase and normal phase liquid chromatography with different types of polysaccharide stationary phases

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ARTICLE INFO

Article history:
Received 12 January 2009
Received in revised form 8 February 2009
Accepted 10 February 2009
Available online 28 February 2009

Keywords:
LC chiral methods
Pharmaceutical related compounds
RPLC
NPLC
Polysaccharide stationary phases

ABSTRACT

The enantioselectivity of a collection of neutral pharmaceutical compounds on six different types of polysaccharide chiral stationary phases (CSPs), Chiralpak AD (and AD-RH), Chiralcel OD (and OD-RH), Chiralpak OJ (and OJ-R), Chiralcel AS (and AS-RH), Sepapak-2 and Sepapak-4 are compared using reversed phase (RPLC) and normal phase liquid chromatography (NPLC). Screening strategies for maximizing the probability of achieving an initial chiral separation hit for neutral compounds using both RPLC and NPLC are described. Further method optimizations are demonstrated by modifying parameters such as organic modifier composition, eluent pH or CSP particle size. Several practical examples of the application of chiral methods for the study of synthetic reaction mixtures are presented. The most critical validation aspects, including limit of detection, specificity, and ruggedness, are also briefly presented.

1. Introduction

Chiral analysis is very important in the pharmaceutical industry since enantiomers of drug compounds may possess quite different pharmacological and toxicological properties. In the past two decades, high performance liquid chromatography (HPLC) with reversed phase (RP) and normal phase (NP) modes have been the most popular techniques used for enantiomeric separations. The applications of these techniques have been extensively covered in recent publications [1–10]. Numerous CSPs have been developed. These CSPs include polysaccharide, Pirkle, protein, crown ether, ligand-exchange, macrocyclic antibiotics, and cyclodextrins [1–10]. Among all of these CSPs, polysaccharide CSPs have been proven to be the most useful. The majority of polysaccharide CSPs are cellulose and amylose derivatives. Enantioselectivity can be varied depending upon the presence of either an ester or carbamate group. These groups can undergo selective interactions with the chiral solutes. These CSPs can be used for both NP and RP modes. Because polysaccharide polymers possess a large number of functional groups, they are able to bind with a wide range of substances through hydrogen bonding, π–π, dipole–dipole, and steric interactions in the NP mode, with hydrophobic interaction also being important in the RP mode [8–15]. Since there are no ionizable groups on the polysaccharide CSPs, the neutral enantiomers are most suitable for use.

In the past two decades, our laboratory has extensively utilized numerous polysaccharide CSPs for a wide range of pharmaceutical compounds, from basic to acidic to neutral. In this report, we compare the separation behaviors of many novel pharmaceutical with both RPLC and NPLC using practical screening first, followed by optimization. A general chiral method development strategy utilizing polysaccharide CSPs together with the successful enantiomeric separation of a number of pharmaceutical related neutral compounds is presented. The practical aspects, especially the use of RPLC for analyzing the mixtures of synthetic organic reaction are unique and useful for the field of drug synthetic chemistry.

2. Experimental

2.1. Reagents

All organic solvents used in the studies were HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Phosphoric acid (85%) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Water used in the study was deionized water purified through a Millipore deionization device (Milford, MA, USA). Chemicals, from compound-C to -N and compound-Q to -T (Fig. 1) were prepared by the Process Research Department of Merck Research Laboratories

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doi:10.1016/j.jpba.2009.02.021

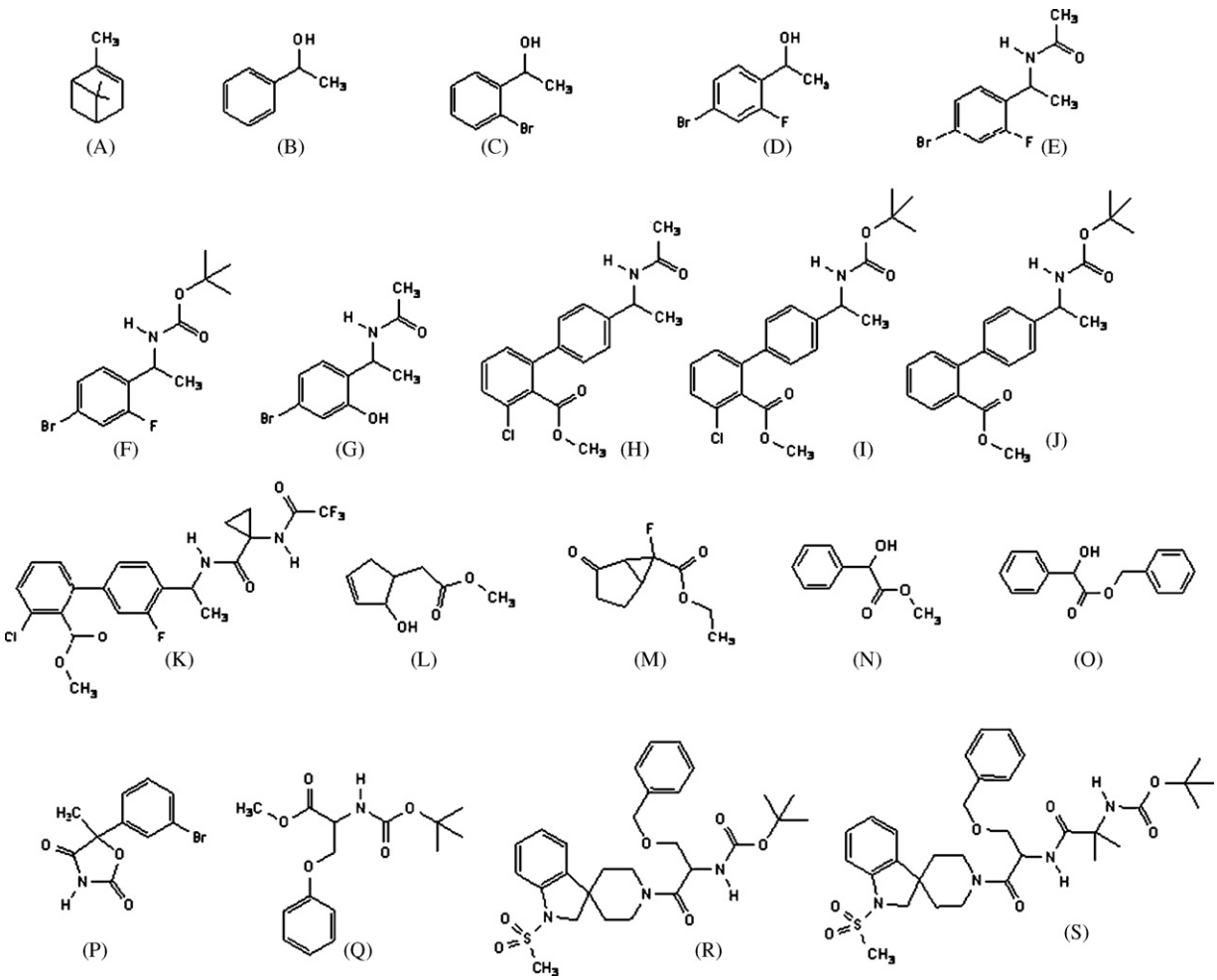


Fig. 1. Structures of studied compounds.

(Rahway, NJ, USA). Compound-A, -B, -N, -O (Fig. 1) were purchased from Aldrich (Milwaukee, WI, USA).

2.2. Instrumentation

An Agilent 1100 system with diode array UV detection and thermal-controller was used. All chromatograms were processed by an Atlas data acquisition system (Version 8.2 Thermo Electron Corporation, PA, USA).

2.3. Chromatographic columns

The columns of Chiralpak AD and Chiralpak AD-RH, Chiralcel OD and Chiralcel OD-RH, Chiralpak OJ and Chiralpak OJ-R, Chiralcel AS and Chiralcel AS-RH were 15 cm in length and 4.6 mm in ID with 5 μm particle size and they were purchased from Chiral Technologies Co. (West Chester, PA, USA). Other columns of Sepapak-1, Sepapak-2 and Sepapak-4 were 15 cm in length and 4.6 mm in ID with 3.0 μm particle size and they were purchased from Sepaserve GmbH (Münster, Germany).

2.4. Chromatographic conditions

All LC separations, except where specified, were performed at a temperature of 25 °C. The mobile phases were gradient for the screening under RPLC mode, and isocratic for NPLC mode. The flow rate was varied from 0.8 to 1.0 mL/min; the injection volume was 10 μL; the detection was UV at 220 nm in all cases. The retention factor k for the two bands was determined as $k = (t_R - t_0)/t_0$, where t_R and t_0 were the retention times of retained and unretained compounds, respectively. In this study, t_0 was estimated based on void marker. The selectivity was calculated as $\alpha = k_2/k_1$.

2.5. Preparation of solutions

The samples were dissolved in isopropyl alcohol. The concentrations of the samples were varied from 0.1 to 1.0 mg/mL.

3. Results and discussion

3.1. Column screening strategy

Fig. 1 shows selected neutral pharmaceutical related compounds, including active pharmaceutical ingredients, synthetic intermediates, and starting materials. All compounds were screened by selected polysaccharide chiral stationary phases under both normal phase and reversal phase conditions. The enantioselectivities of each compound with various columns and mobile phase conditions are listed in Table 1.

Six stationary phases including Chiralpak AD (and AD-RH), Chiralcel OD (and OD-RH), Chiralpak OJ (and OJ-R), Chiralcel AS (and AS-RH), Sepapak-2 and Sepapak-4 were selected for the screening under normal phase mode. These six stationary phases are known to show high chiral recognition abilities for various chi-

Table 1
Screen results for enantioseparations of studied compounds on various polysaccharide-based columns.

ID	Enantioselectivity (α) of the optimum hits during screening (baseline separation are achieved at $\alpha \geq 1.15$)											
	AD		OD		AS		OJ		Sepapak-2		Sepapak-4	
	NP	RP	NP	RP	NP	RP	NP	RP	NP	RP	NP	RP
A	1.00[a–f]	1.0[g]	1.10[c]	1.2[g]	1.25[a]	1.0[g–j]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.1[g]	1.00[a–f]	1.0[g]
B	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.27[h]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g]	1.00[a–f]	1.07[h]	1.00[a–f]	1.15[h]
C	1.03[d]	1.1[g]	1.00[a–f]	1.0[g–j]	1.05[d]	1.0[g]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g–j]
D	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g]	1.00[a–f]	1.0[g]	1.00[a–f]	1.0[g]	1.00[a–f]	1.0[g–j]	1.17[e]	1.10[g]
E	1.20[e]	1.20[g]	1.00[a–f]	1.09[i]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.24[g]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g–j]
F	1.03[e]	1.1[g]	1.05[a]	1.0[g]	1.23[a]	1.2[g]	1.00[a–f]	1.1[g]	1.02[c]	1.0[g]	1.00[a–f]	1.0[g]
G	1.03[e]	1.2[g]	1.05[a]	1.09[h]	1.23[a]	1.2[g]	1.00[a–f]	1.1[g]	1.02[c]	1.0[g–j]	1.00[a–f]	1.0[g–j]
H	1.02[e]	1.1[g]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g–j]	1.20[e]	1.0[g–j]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g–j]
I	1.12[e]	1.3[g]	1.23[a]	1.01[h]	1.20[a,e]	1.0[g–j]	1.00[a–f]	1.0[g–j]	1.05[a]	1.0[g–j]	1.00[a–f]	1.0[g–j]
J	1.03[e]	1.2[g]	1.00[a–f]	1.0[g–j]	1.12[a,e]	1.02[j]	1.00[a–f]	1.0[g–j]	1.21[a]	1.06[h]	1.17[a]	1.08[h]
K	1.00[a–f]	1.0[g–j]	1.35[h]	2.31[h]	1.00[a–f]	1.02[j]	1.00[a–f]	1.0[g–j]	1.22[a]	1.10[h]	1.08[a]	1.00[g–j]
L	1.23[a]	1.39[h]	1.00[a–f]	1.0[g]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g–j]	1.01[a]	1.00[g–h]	1.00[a–f]	1.00[g–j]
M	1.25[a]	1.78[g]	1.00[a–f]	1.0[g–j]	1.02[d]	1.0[g]	1.00[a–f]	1.08[g]	1.02[a]	1.00[g–h]	1.00[a–f]	1.00[g–j]
N	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.15[i]	1.00[a–f]	1.01[i]	1.00[a–f]	1.0[g–j]	1.04[a]	1.14[i]	1.00[a–f]	1.00[g–j]
O	1.00[a–f]	1.0[g–j]	1.00[a–f]	4.21[i]	1.75[a]	1.01[i]	1.11[e]	1.05[g]	1.02[a]	1.10[i]	1.00[a–f]	1.00[g–j]
P	1.00[a–f]	1.0[g–j]	1.21[a]	1.49[i]	1.00[a–f]	1.0[g–j]	1.14[e]	1.06[g]	1.15[a]	1.08[g]	1.07[e]	1.00[g–j]
Q	1.00[a–f]	1.0[g–j]	1.25[a]	1.05[i]	1.00[a–f]	1.0[g–j]	1.00[a–f]	1.0[g–j]	1.02[a]	1.00[g–h]	1.00[a–f]	1.00[g–j]
R	1.09[d]	1.18[g]	1.37[f]	1.28[h]	1.36[f]	1.0[g]	1.00[a–f]	1.0[g–j]	1.05[c]	1.07[g]	1.24[f]	1.06[g]
S	1.00[a–f]	1.0[g–j]	1.29[f]	1.15[j]	1.00[a–f]	1.0[g]	1.00[a–f]	1.0[g–j]	1.03[c]	1.06[g]	1.21[f]	1.13[j]

Mobile phases—a: 10/90 IPA/hexane; b: 50/50 IPA/hexane; c: 75/25 IPA/hexane; d: 75/25 EtOH/hexane; e: 10/90 EtOH/hexane; f: 50/50 EtOH/hexane; g: 70/30 H_2O/MeCN to 30/75 H_2O/MeCN gradient; h: 70/30 H_2O/IPA to 30/75 H_2O/IPA gradient; i: 70/30 H_2O/MeOH to 30/75 H_2O/MeOH gradient; j: 70/30 H_2O/EeOH to 30/75 H_2O/EeOH gradient.

ral compounds. Considering solvent miscibility, hexane with IPA and hexane with ethanol were chosen as mobile phases. Since the equilibrium of the normal phase chromatographic process is slow, an isocratic mobile phase elution is preferred. The drawback of the isocratic elution is that the mobile phase ratio is fixed. At such conditions, some compounds may be not eluted within a time frame or eluted within the void time period (t_0), since the retention time of an individual compound is a function of its structural features and mobile phase ratio. However, it is recognized that the retention time of each individual compound is similar on different polysaccharide chiral stationary phases within a reasonable window. Therefore, before the full screen of a new compound has taken place, a suitable mobile phase ratio for the full column screen can be selected through a quick test on one of these columns.

A reversed phase mode with six columns (as described above) was also selected. Since all polysaccharide stationary phases and selected compounds are neutral, a simple mobile phase containing water and an organic modifier is sufficient. Acetonitrile is chosen as an organic modifier for the primary screen due to its low viscosity and low UV-cut-off. Other common organic solvents including methanol, ethanol, and isopropyl alcohol are also used for comparison in this study. In practice, such organic modifiers would be used only if no hit is achieved by acetonitrile. Simple gradient profiles (shown in Table 1) are used.

As shown in Table 1, separation hits were achieved for all selected compounds after the screening. Some compounds could be separated on multiple columns, but none of the selected compounds was separated on every selected column. Reversal of the elution order occasionally occurred from column to column or from NP to RP. Such reversals occurred more often with different columns than with different mobile phase modes. It is well known that each polysaccharide stationary phase contains different chiral recognition sites that may interact with various functional groups on the enantiomeric compounds differently. However, it is difficult to predict a suitable column for each individual enantiomeric compound. Therefore, a quick screen is the most appropriate and efficient way to find a starting point for developing a suitable chiral method. The results shown in Table 1 are good evidence of this fact.

3.2. Method optimizations

Once the initial separation is obtained through screening, optimization can be achieved by modifying parameters such as polar modifier type, organic modifier type, temperature, column particle size, etc. for NP mode. Optimization can also be achieved by modifying buffer pH, organic modifier type, temperature and particle size, etc. for RP mode. Many optimization aspects are well known. Therefore, only items that have not been reported before were presented.

3.2.1. Effect of mobile phase additive for NPLC

It is well known that the addition of polar mobile phase additives such as triethylamine (TEA) and trifluoroacetic acid (TFA) to the mobile phase are necessary for the separation of basic or acidic enantiomers. Our studies have shown that the addition of such additives can also help separation efficiency for even a select group of neutral compounds. Fig. 2 shows an example. Compound-S contains multiple nitrogen atoms (the basic site was protected by BOC group). The peaks on the top chromatogram were obtained using mobile phase with IPA/hexane while the peaks on the bottom chromatogram were obtained using the same ratio of IPA/hexane with 0.1% TEA. The peak shapes of the latter chromatogram are much sharper than those of the former chromatogram. It is well accepted that chiral recognition on the polysaccharide-based CSPs under NPLC is mainly due to hydrogen bonding and dipole–dipole interaction between enantiomeric solutes and CSPs. There are multiple non-specific hydrogen bonding among the multiple side-chains on compound-T and the stationary phases, which result in peak-broadness. TEA can compete with such non-enantiospecific interactions and thus improve peak shape.

3.2.2. Effect of mobile phase compositions for RPLC

For RPLC, water or phosphate buffers with acetonitrile are the best mobile phase compositions in terms of peak efficiency and method sensitivity. These combinations offer better solute mass transfers and lower UV cut-offs, and thus better LODs. For most pharmaceutical compounds, the LOD is often achieved at as low as 0.05% with such mobile phase combinations. Therefore, if separation hits are achieved by a mobile phase composed of acetonitrile

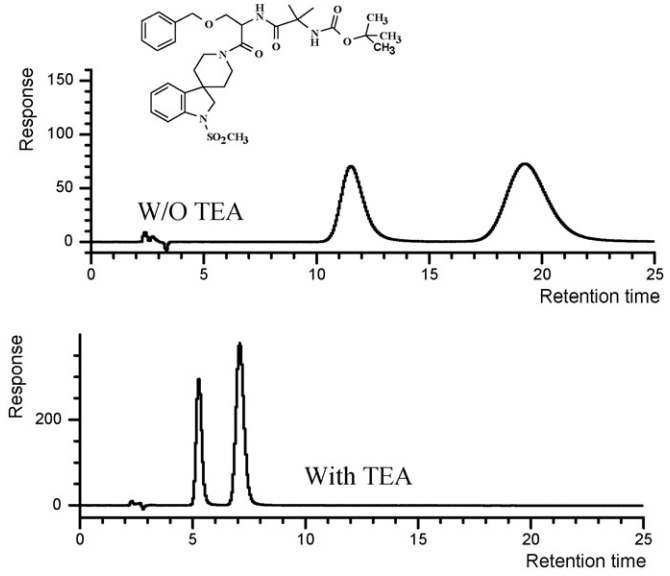


Fig. 2. Effect of mobile phase additive for separation of compound-S on NPLC. Experimental conditions: column: Sepapak-1. Mobile phase for top chromatogram: IPA/hexane, ratio: 75/25 (v/v); mobile phase for bottom chromatogram: IPA/hexane/TEA, ratio: 75/25/0.1 (v/v/v). Flow rate: 0.8 mL/min. UV wavelength: 220 nm. Column temperature: 25 °C. Injection volume: 10 μL.

and water (or phosphate buffer) but the resolutions of these hits are not good enough, optimization should focus on achieving baseline resolutions by varying the ratio of the mobile phase. An isocratic run is often helpful. If there is no hit at all with the water (or phosphate buffer)–acetonitrile mobile phase, additional screens with other organic modifiers can be performed. For comparison, other common organic modifiers, including acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), and isopropyl alcohol (IPA) were screened for all selected compounds on Chiralpak AD-RH, Chiralcel OD-RH, Chiralpak OJ-R, Chiralcel AS-RH, Sepapak-2 and Sepapak-4 columns (Table 1). It was found that peak shape is sharper with less retention time when MeCN is used for all selected columns. The retentions of the solutes increase in the order of MeOH > EtOH > IPA > MeCN in most cases. Such behavior is usually observed on achiral columns and is attributed to increasing viscosity or solvation through hydrogen-bonding with alcoholic solvents. However, the behaviors of IPA in several cases are different. As can be seen from Fig. 3, the enantiomeric separation of compound-L with IPA is much better than the enantiomeric separations of compound-L with all other modifiers.

The effect of the concentration of MeCN on the retention factor of compound-B in the range from 10% to 45% was studied. The retention factors for both enantiomers of compound-B decreased as the ratio of MeCN increased. However, plots of log k vs. % MeCN were not linear (Fig. 4) compared to typical RPLC chromatographic behavior. Such non-linear trends may be due to additional non-hydrophobic interactions between enantiomers and stationary phases, even though the separation is under RPLC mode. These additional diastereomeric formations between enantiomers and coated chiral selectors may undergo hydrogen-bonding and stereo-inclusion. In the most cases, the optimum ratio of acetonitrile is within 10–30% range.

3.2.3. *Effect of pH for RPLC*

Since both polysaccharide CSPs and the solutes used in the study are neutral, changes in mobile phase pH might be expected to have little or no effect on the enantiomeric separations. However, real samples often contain complex matrixes, making changes in eluent pH is a useful tool for method development optimization.

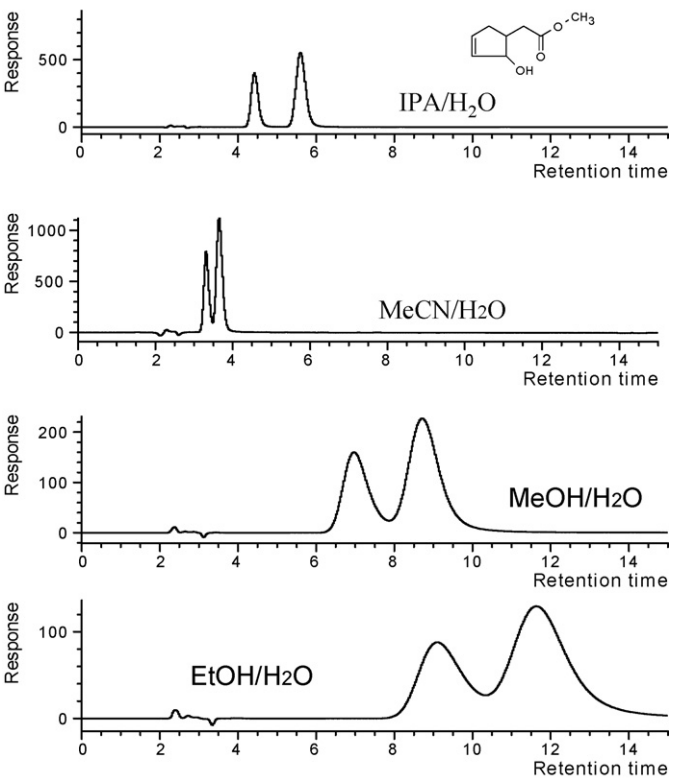


Fig. 3. Effect of organic modifiers for separation of compound-L on RPLC. Experimental conditions: column: Chiralpak AD-RH (15 cm in length and 4.6 mm in ID with 5 μm particle size). Mobile phase for each chromatogram (from top to bottom): (1) IPA/H_2O, (2) MeCN/H_2O, (3) MeOH/H_2O and (4) EtOH/H_2O; ratio for all mobile phases: 10/90 (v/v). Flow rate: 0.8 mL/min. UV wavelength: 220 nm. Column temperature: 25 °C. Injection volume: 10 μL.

Fig. 5 shows an example. Compound-M was prepared through an asymmetric synthesis. A reaction sample contained copper triflate carried over from a previous reaction and would not be eluted out with either water or higher pH mobile phases within the desired run time. The consequence of this was that either the column life would suffer or the potential peak interferences would occur from run to run. However, with a low pH mobile phase, this solute was washed out from each run. Therefore, it is recommended that a phosphate buffer with pH 2.1 be used as the mobile phase for the analysis of the reaction mixture of compound-M.

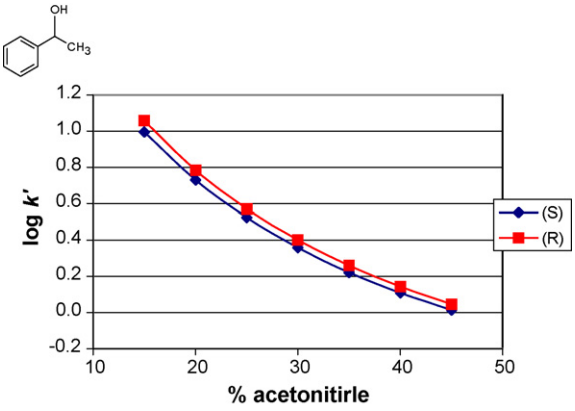


Fig. 4. Effect of organic modifiers for separation of compound-B on RPLC. Experimental conditions: column: Chiralpak OJ-R (15 cm in length and 4.6 mm in ID with 5 μm particle size). Mobile phase conditions are varied.

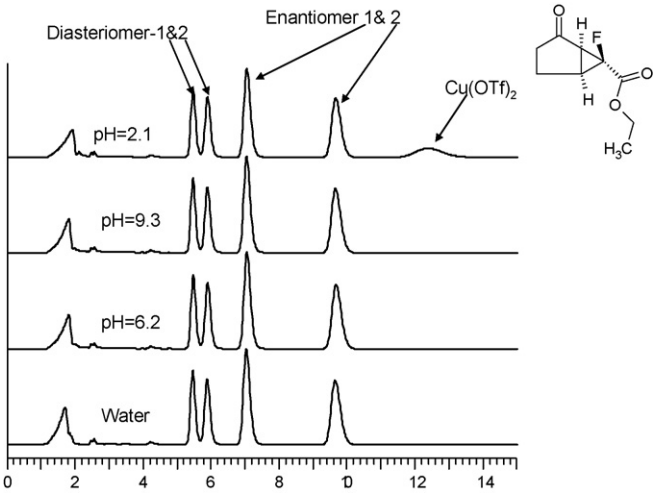


Fig. 5. Effect of mobile phase pH on the sample matrixes for separation of compound-M on RPLC. Experimental conditions: column: Chiralpak AD-RH (15 cm in length and 4.6 mm in ID with 5 μm particle size). Mobile phase: MeCN/phosphate buffer, pHs are varied. Flow rate: 1.0 mL/min. UV wavelength: 220 nm. Column temperature: 25 °C. Injection volume: 10 μL.

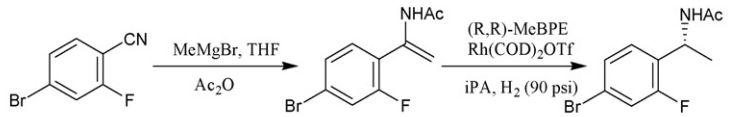
3.3. Method validation aspects

It is important that developed chiral methods can be validated. Many validation items applied in chiral separation methods, such as precision, accuracy, linearity, etc. are similar to those applied in achiral separation methods. The most critical validation challenges for chiral separation methods are limit of detection (LOD), specificity, and ruggedness when a complicated sample matrix is used.

3.3.1. Comparison of NPLC and RPLC for limit of detection

The screening results for the selected 19 neutral compounds showed a hit rate of 53% for RPLC vs. a hit rate of 42% for NPLC. The selectivity between two modes is essentially the same. The case by case comparisons of the data presented in Table 1 suggests that NPLC and RPLC are complementary techniques. This sub-section is focused on pros and cons of the two modes from a validation perspective.

From a validation perspective, there are certain pros and cons of the normal phase and reversed phase approaches that must be considered. In general, the peak shape obtained from RPLC is much more efficient than the shape obtained from NPLC under concentration ranges <1 mg/mL. However, NPLC allows for the ability to load samples as concentrated as 2–3 mg/mL without sacrificing much resolution. Such large concentrations would cause peak broadening and hence reduce the resolution achieved with RPLC. Common practice for the determination of minor enantiomeric impurities of a pharmaceutical related compound is based on area percent. The desired LOD can be as low as 0.05–0.1%. Such low LOD requirements are challenges for both NPLC and RPLC. However, under RPLC, LOD difficulties can be overcome with higher peak efficiency and lower UV wavelengths. We have previously reported a comparison signal to noise (S/N) ratios at wavelengths of 200 and 220 nm using different buffers, including sodium phosphate, triethyl amine-phosphate, triethyl amine-acetate, and triethyl amine-citrate on CE [16]. The results clearly demonstrated that the phosphate buffer has



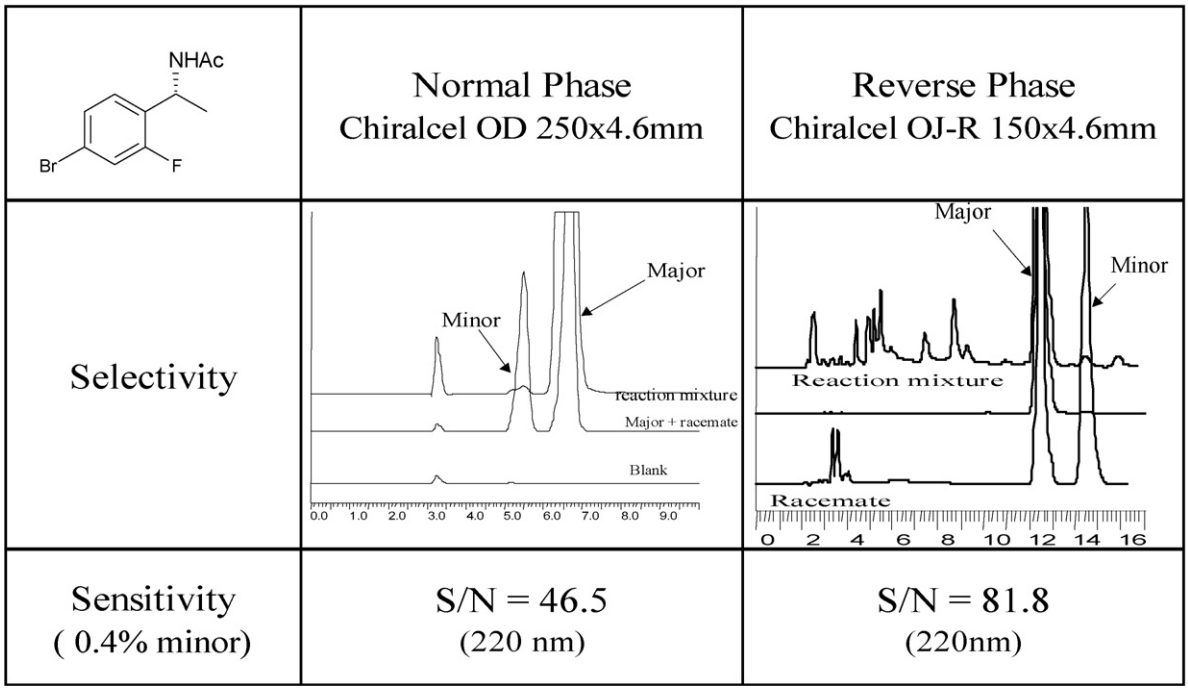


Fig. 6. Comparison of NPLC and RPLC for method specificity and sensitivity of compound-E in a reaction mixture of an asymmetric synthesis. Experimental conditions: for NPLC mode: column: Sepapak-1 (15 cm in length and 4.6 mm in ID with 3.0 μm particle size); mobile phase: IPA/hexane, ratio: 10/90 (v/v); for RPLC mode: column: Chiralpak OJ-R (15 cm in length and 4.6 mm in ID with 5 μm particle size); mobile phase: 0.1% H_3PO_4/MeCN, ratio: 65/35. For both modes: flow rate: 1.0 mL/min. UV wavelength: 220 nm. Column temperature: 25 °C. Injection volume: 10 μL.

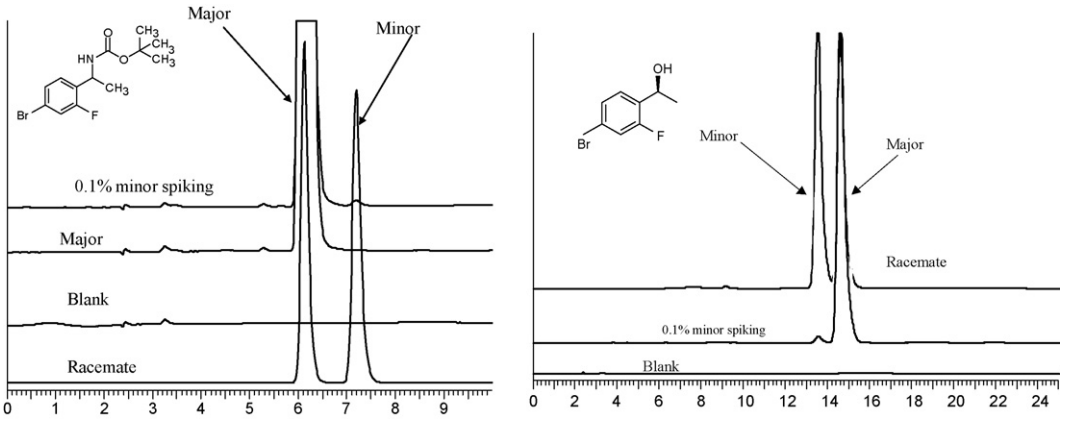


Fig. 7. Effect of elution order on LOD of compound-D. Left chromatograms, column: Chiralpak AD-RH (15 cm in length and 4.6 mm in ID with 5 μm particle size), mobile phase: MeCN/H_2O, 35/65. Right chromatograms, column: Chiralpak OJ-R (15 cm in length and 4.6 mm in ID with 5 μm particle size), mobile phase: MeCN/H_2O, 15/85. Flow rate: 1.0 mL/min, UV wavelength: 220 nm. Column temperature: varied. Injection volume: 10 μL.

the highest S/N ratio. Therefore, it is recommended utilizing phosphate buffers combined with acetonitrile as the RPLC mobile phase when UV detection is required.

Another advantage of using RPLC is that is a technique comparable with LC–MS. It has been become more and more common practice to detect the enantiomers by LC–MS when isolated individual enantiomer is not available or if some achiral impurities are co-eluted with enantiomers. Since the phosphate buffers will cause clogging on MS detector, it is recommended using the volatile buffers when MS detection is required.

3.3.2. Comparison of NPLC and RPLC for method specificity

Another concern is the ability to apply the method for complicated sample matrixes. During the synthesis of pharmaceutical compounds, reaction mixtures often contain polar species (as mentioned in the previous sections). These polar contents are often stuck on the column or co-elute with peaks of enantiomers under NPLC mode. Fig. 6 shows a separation example for compound-E with a reaction mixture sample. The RPLC mode is clearly shown as a superior mode. RPLC also provided a higher signal to noise ratio than NPLC did under the same low sample concentration of 0.5%.

3.3.3. Effect of elution order on LOD

In general, it is preferred to have a minor enantiomer elute in the front of the major enantiomer so as to avoid interference from the tail of the major enantiomer. However, the peaks of most neutral compounds separated by polysaccharide CSPs are fairly symmetrical, especially under RPLC mode. Therefore, to have front minor elution is vital only if resolution is limited. Fig. 7 demonstrates both cases. A LOD of 0.1% for both methods was verified by spiking minor enantiomer to the samples.

3.3.4. Ruggedness

The variations in retention time and the resolutions of the chiral method with polysaccharide CSPs were noted from day to day and from column to column. Such variations can be reduced by designating mobile phase compositions and columns to specific compounds, and by periodically flushing the columns with acidic or basic mobile phases to remove any buildup from the sample matrix. The enantioseparation of all 19 neutral compounds presented in Table 1 were validated and showed adequate ruggedness.

4. Conclusions

A strategic approach to the development of LC chiral methods for neutral pharmaceutical related compounds using normal phase and reversed phase liquid chromatography with different types of polysaccharide stationary phases was demonstrated. Practical chiral separation methods for neutral pharmaceutical compounds can be developed through initial screening on different polysaccharide chiral stationary phases under both NPLC and RPLC, then can be further optimized and validated.

Acknowledgment

The authors wish to thank Merck Process Research Department for providing the studied compounds.

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