1
Method Development and Optimization of Enantioseparations Using Macrocyclic Glycopeptide Chiral Stationary Phases

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1.1
Introduction

The efficient development of enantiomeric separations has become increasingly important, especially in the pharmaceutical industry, as optical isomers often produce different biological properties, some detrimental to further drug development. The closer to the point of drug discovery these issues are resolved, the less costly the outcome will be. This recognition has put pressure on the demand for more efficient chiral screening protocols. The analysis and preparation of a pure enantiomer often involve resolution from its antipode. Among all the chiral separation techniques, chiral high-performance liquid chromatography (HPLC) and mass spectrometry (MS) have proven to be the most robust and widely applicable platform. Chiral stationary phase (CSP) development has plateaued, but several CSPs now dominate selectivity screening protocols.

Currently, several hundred CSPs have appeared in publications and over 110 of them are available commercially [1]. These CSPs are made by using either a polymeric structure or a small ligand (MW < 3000) as the chiral selector. The polymeric CSPs include synthetic chiral polymers [2] and naturally occurring chiral structures [3–5]. The most commonly used natural polymers include proteins and carbohydrates (cellulose and amylose). The chiral recognition mechanisms for these polymeric CSPs are relatively complicated. A protein, for example, is often complex enough to contain several chiral binding sites, in which case the major (high-affinity) site may differ for any given pair of enantiomers [6]. The other types of CSPs, with small molecule as the chiral selector, include ligand-exchange CSPs [7], π-complex (Pirkle-type) CSPs [8, 9], crown ether CSPs [10], cyclodextrin CSPs [11–15] and macrocyclic glycopeptide CSPs [16–20]. Compared with the polymeric CSPs, the separation mechanisms on these small-molecule CSPs are better characterized and understood. Macrocyclic glycopeptides, which were introduced by Armstrong in 1994, are one of the newest classes of CSPs [44]. To date, there are six macrocyclic glycopeptides CSPs available commercially [20] – vancomycin (V and V2), teicoplanin (T and T2), teico-

Chiral Separation Techniques. Third Edition. Edited by G. Subramanian

ISBN: 978-3-527-31509-3

planin aglycone (TAG) and ristocetin A (R). Much research effort has been devoted to the characterization and application of these CSPs for a wide variety of chiral compounds.

1.2 Structural Characteristics of Macrocyclic Glycopeptide CSPs

1.2.1 Chiral Recognition Mechanisms

The macrocyclic glycopeptides vancomycin, teicoplanin and ristocetin A are produced as fermentation products of *Streptomyces orientalis*, *Actinoplanes teichomyceticus* and *Nocardia lurida*, respectively. All three of these related compounds consist of an aglycone "basket" made up of fused macrocyclic rings and a peptide chain with differing numbers of pendant sugar moieties off the phenoxide groups (Fig. 1.1). The macrocyclic rings of vancomycin and teicoplanin contain two chloro-substituted aromatic rings whereas the analogous portion of ristocetin A has no chlorine substituents.

Vancomycin is the smallest of the three basic molecules, consisting of three macrocyclic rings and a glycoside comprising D-glucose and L-vancosamine. The other two glycopeptides are larger, having four fused rings and different types of pendant sugar moieties. Teicoplanin has three monosaccharides: one D-mannose and two D-glucosamines. On one of the latter sugars was attached a hydrophobic acyl side-chain (hydrophobic tail). Ristocetin A has a pendant tetrasaccharide (arabinose, mannose, glucose and rhamnose) and two monosaccharide moieties (mannose and ristosamine) [21]. In addition to the natural CSPs, teicoplanin aglycone was produced by removing the sugar moieties from teicoplanin. The structural characteristics of the four basic macrocycles are outlined in Table 1.1. In addition, V2 and T2 were produced using different bonding chemistries on the surface of the silica compared with V and T, respectively. Although the chemical ligand remains the same, the loading and accessibility of the key interaction sites are different between V and V2 [22] and T and T2, yielding higher selectivity and sample loading capacity for certain significant classes of compounds.

All macrocyclic glycopeptides have analogous ionizable groups which have been proven to play a major role in their association with ionizable analytes and, thus, chiral recognition. For example, there is an amino group on the aglycone portion of each CSP. There is a carboxylic acid moiety on the other side of macrocyclic basket of both vancomycin and teicoplanin, while the equivalent group on ristocentin A is methylated. When the sugars are removed from teicoplanin, a dramatic increase in selectivity is observed for a number of types of racemates [23]. This variety of structures and functionalities on the macrocyclic glycopeptides provides a unique range of interactions for chiral recognition. A list of available interactions and their relative strengths is given in Table 1.2.

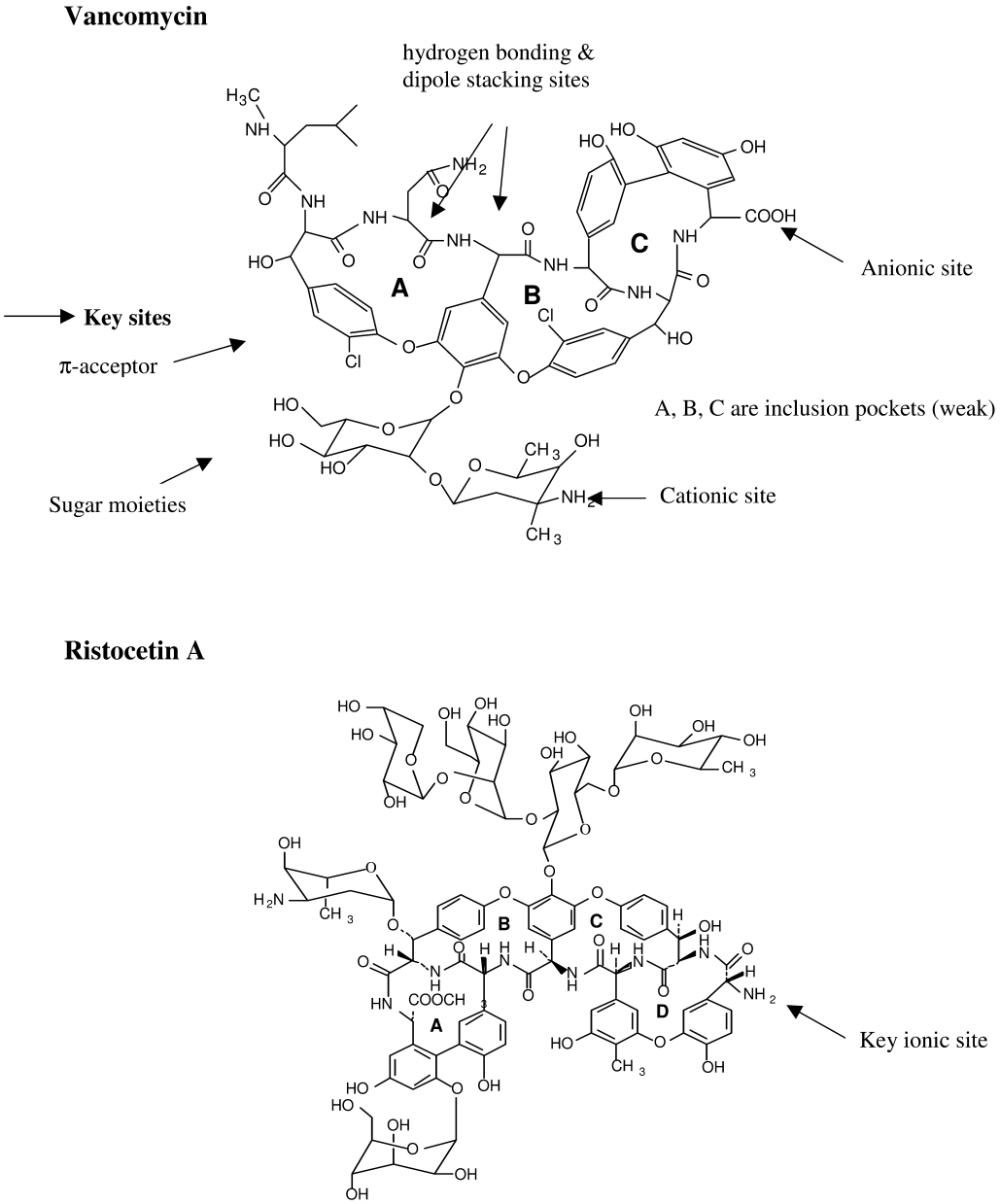
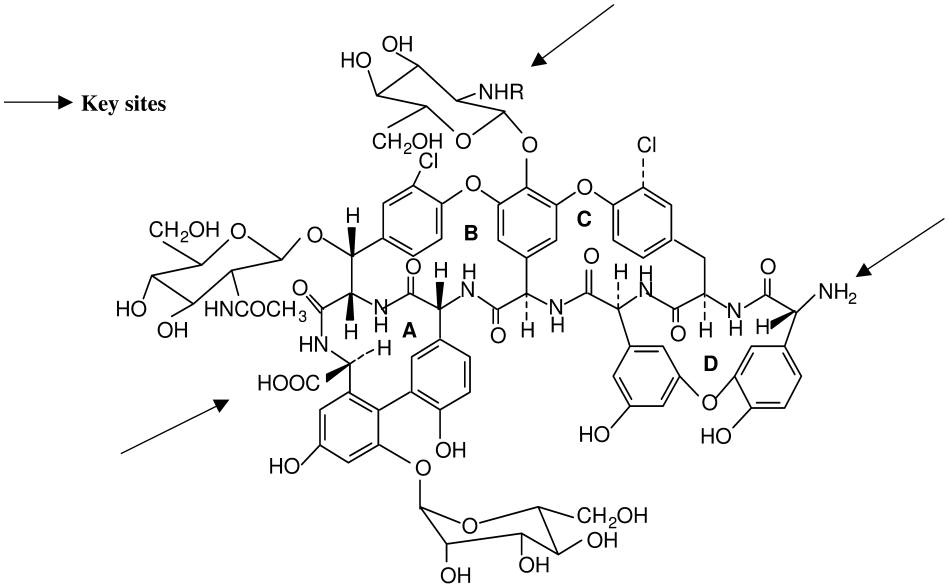


Fig. 1.1 Proposed structures of glycopeptide CSPs.

Teicoplanin



Teicoplanin Aglycone

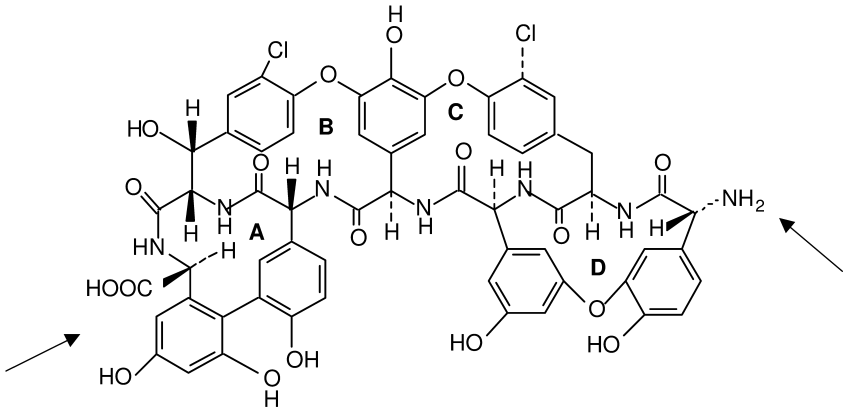


Fig. 1.1 (continued)

Table 1.1 Structural characterics of macrocyclic glycopeptide chiral ligands.

	Vancomycin	Teicoplanin	Ristocetin A	Teicoplanin aglycone
Molecular weight	1449	1877	2066	1197
Stereogenic centers	18	23	38	8
Macrocycles	3	4	4	4
Sugar moities	2	3	6	0
Hydroxyl groups	9	15	21	7
Amino groups	2	1	2	1
Carboxyl groups	1	1	0	1
Amido groups	7	7	6	7
Aromatic groups	5	7	7	7
Methyl esters	0	0	1	0
Hydrophobic tail	0	1	0	0
p*I* value	7.2	3.8–6.5	7.5	N/A

Table 1.2 Relative strength of potential interactions between macrocyclic glycopeptide CSPs and chiral analytes.

Anionic or cationic interactions	Very strong
Hydrogen bonding	Very strong
π–π complexation	Strong
Steric interactions	Medium strong
Inclusion complexation	Medium
Dipole stacking	Weak

1.2.2 Multi-modal Chiral Stationary Phases

From the structural information given above, it can be seen that the macrocyclic glycopeptide CSPs are multi-modal such that a variety of mobile phase types can be used to initiate selectivity [16–18]. Typically, these mobile phase systems are classified as polar ionic mode (PIM, nonaqueous), reversed-phase mode (RP, aqueous), polar organic mode (POM, nonaqueous) and normal-phase mode (NP, nonaqueous). Since these macrocyclic glycopeptides are covalently bonded to silica gel through multiple (>4) linkages, there is no detrimental effect when switching from one mobile phase system to another. The only limitation is the pH range of the aqueous buffer, which should be between 2.8 and 7.0. The enantioselectivities of these CSPs are different in each of the mobile phase systems, because certain molecular interactions (between CSP and analyte) function more effectively in certain eluent conditions. Table 1.3 shows the breakdown of separation mechanisms versus the mobile phase systems in descend-

Table 1.3 Possible separation mechanisms for three types of mobile phase systems on the macrocyclic glycopeptide CSPs.

Polar ionic mode	Ionic interaction Hydrogen bonding Steric interaction π–π interaction
Reversed-phase mode	Ionic interaction Hydrogen bonding Inclusion complexation Steric interaction
Polar organic/normal-phase mode	Hydrogen bonding π–π interaction Steric interaction Dipole stacking

ing order of strength. Statistically, the most successful mobile phase for pharmaceutical compounds is the nonaqueous PIM on macrocyclic glycopeptide CSPs. This mode accounted for more than 50% of the applications, balanced by the RP mode, while the POM and NP mode resulted in about 15% of separations. The most unique characteristic of these CSPs is that they have effective chiral ionic interaction sites on either side of the aglycone: vancomycin has a secondary amine and a carboxyl group, teicoplanin and teicoplanin aglycone have a primary amine and a carboxyl group whereas ristocetin A has one primary amine only. These ionic sites provide the key interaction site for any compound with ionizable groups. Since chiral separations require three-point simultaneous interactions, the subtle differences between these CSPs near the anchoring site provide complementary separation effects.

1.3 Enantioselectivity as a Function of Molecular Recognition

1.3.1 Ionizable Molecules

1.3.1.1 Polar Ionic Mode

The PIM is a preferred mobile phase system to take advantage of ionic interactions efficiently. This mobile phase has beneficial MS-compatible components and low volatility and is easy to manipulate. When dealing with ionizable compounds (either acid or base), the proximity and availability of functional groups around the chiral center control the degree of selectivity/separation. For example, when propranolol was first separated using the PIM on a teicoplanin column, most β-blockers were also found to be baseline-resolved by the same mobile phase. These amino alcohols have identical key functionalities around the chiral center

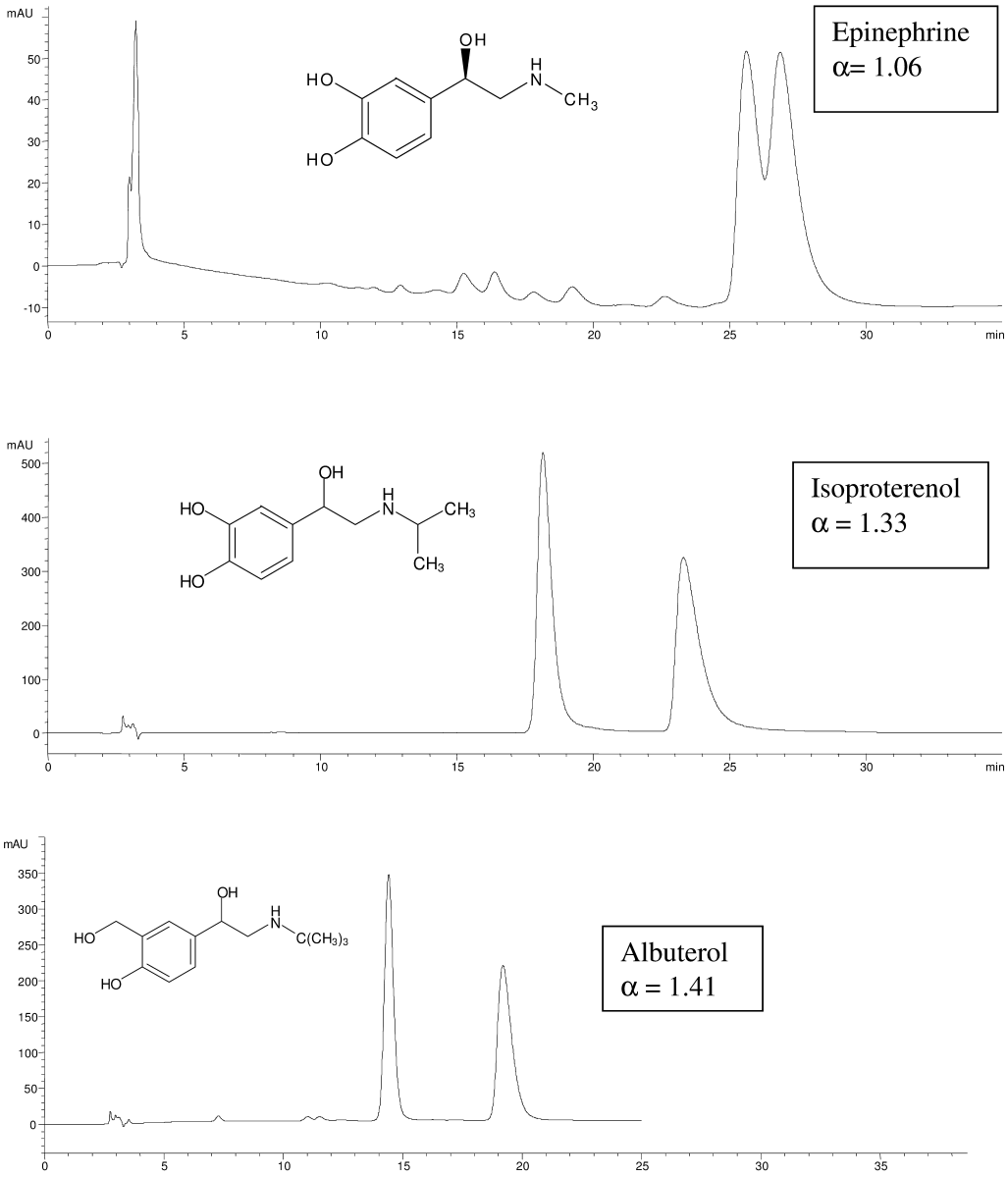


Fig. 1.2 Selectivity comparison for structurally related amino alcohols using a teicoplanin column in the polar ionic mode. Mobile phase, 100:0.1:0.1 MeOH–HOAc–TEA; flow-rate, 1 mL min^{-1}; UV detection at 230 nm.

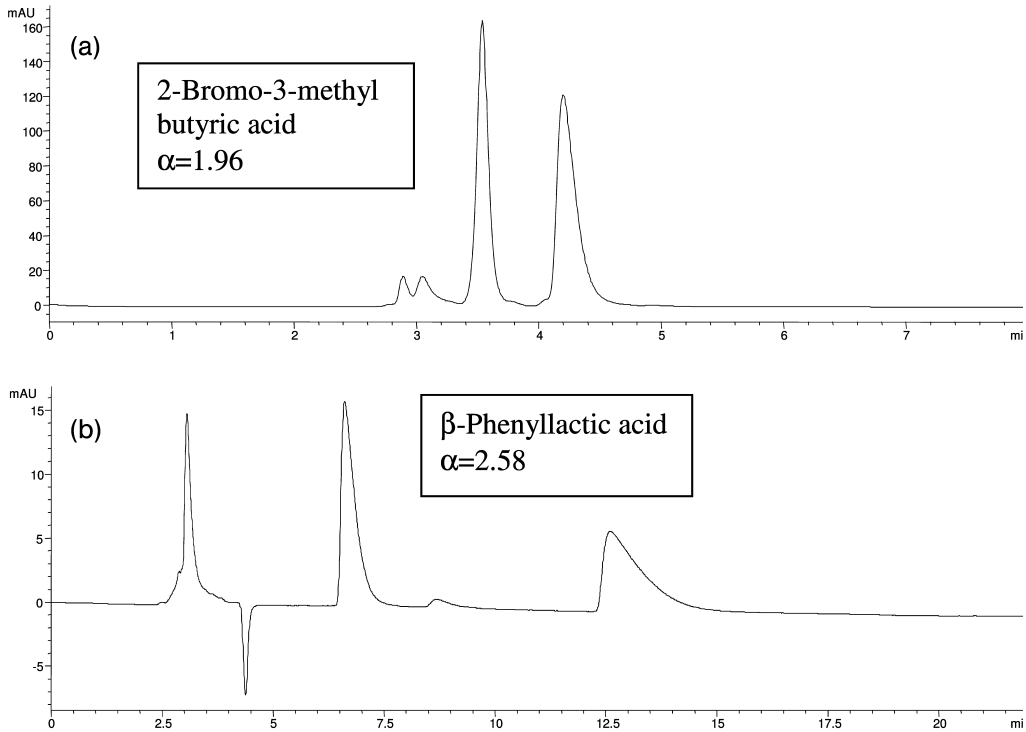


Fig. 1.3 Enantiomeric separation of *α*-hydroxy-/halogenated acids on ristocetin CSP. Column, 250×4.6 mm i.d.; mobile phase, 100:0.1 MeOH–NH_4OH; flow-rate, 1 mL min^{-1}; UV detection at 230 nm. (a) 2-Bromo-3-methylbutyric acid; (b) *β*-phenyllactic acid.

(secondary amine and a hydroxyl plus an aromatic moiety). The carboxyl group (COO^-) of the teicoplanin provided the anchoring point with the amino group (secondary $-NH^+$) of the *β*-blocker. However, the degree of selectivity obtained was dictated by the bulkiness of alkyl groups off the anchoring site (secondary $-NH^+$). The best examples to demonstrate this were albuterol, isopreterol and epinephrine, as their structures are very similar. Note the decreased selectivity that is observed in Fig. 1.2, from albuterol (*tert*-butyl group) to isoproterenol (isopropyl group) to epinephrine (methyl group). It follows that steric effects play a significant role in chiral selectivity in the PIM system.

The predictability of selectivity is further shown with *α*-hydroxy-/halogenated carboxylic acids on a ristocetin A column. Again, the mobile phase is a PIM (Fig. 1.3). In this example, a carboxylic group of the analyte initiates the interaction with the amino group of the ristocetin A chiral stationary phase. Then, an H-bonding-capable functional group (bromine or/hydroxyl) enhances the chiral recognition. The last point of interaction (minor one), which is steric or hydrophobic, completes the enantioselective interactions. Note that in the PIM, the eluent is

mostly methanol, which has a strong H-bonding capability. With this mobile phase system, only ionic and H-bond interactions between the CSP and analyte stand out and interact with each other more effectively, leading to retention and possible separation. Most profen-type compounds can be separated in a similar fashion, but only with the ristocetin A CSP. Again, the selectivity is dictated by the availability and the strength of the additional functionalities (e.g. H-bond, dipole) in addition to carboxyl group and aromatic rings. It is not surprising that ibuprofen demonstrates no selectivity in the PIM since it has only a hydrocarbon functional group (off the aromatic ring) that will not provide significant interaction in this mobile phase system. Finally, it should be noted that the effectiveness of these chiral interactions is inversely proportional to the distance from the chiral center of the analyte. In other words, the shorter the distance of the chiral interactions to the chiral center is, the higher the selectivity will be.

1.3.1.2 **Reversed-phase Mode**

The typical RP mode involves the use of aqueous buffers as part of the mobile phase composition. However, macrocyclic CSPs can tolerate from 0 to 95% buffer without any deleterious effects. In this mobile phase system, ionic and H-bond interactions and hydrophobic inclusion complexation may provide the needed mechanisms for chiral recognition. For ionizable compounds (acid or base), the anchoring point is still either carboxyl or amino group, respectively. Then, H-bond and hydrophobic/inclusion complexation helps complete the chiral discrimination of the analyte. There are two reasons why ketoprofen was separated better in the RP mode than in the PIM on the ristocetin A column. First, in the PIM, the carbonyl group of the analyte is far away from the chiral center so that the effectiveness of H-bond interaction is compromised. Second, in the RP mode, the aromatic ring helps stabilize the molecule through inclusion complexation within the cavity of the CSP so that H-bonding with carbonyl becomes more effective. Another example is α-methylbenzylamine, separated on a vancomycin (V2) CSP. When the PIM was used initially, just baseline separation was obtained. When water was added to the mobile phase, the selectivity, along with separation, increased (Fig. 1.4). By adding water, the structural conformation of CSP changes such that it favors inclusion complexation, leading to a much better separation.

1.3.2 **Neutral Molecules**

For neutral molecules, the chiral recognition processes rely heavily on the peptide chain and the multiple cavities on the cleft of the CSPs. Therefore, in the RP mode, in addition to the availability of inclusion complexation, analytes should have multiple H-bond donor/acceptor sites for a decent separation, although it is more unpredictable than for ionizable compounds. Compounds without an ionizable group (neutral) are also suitable for POM/NP systems. In

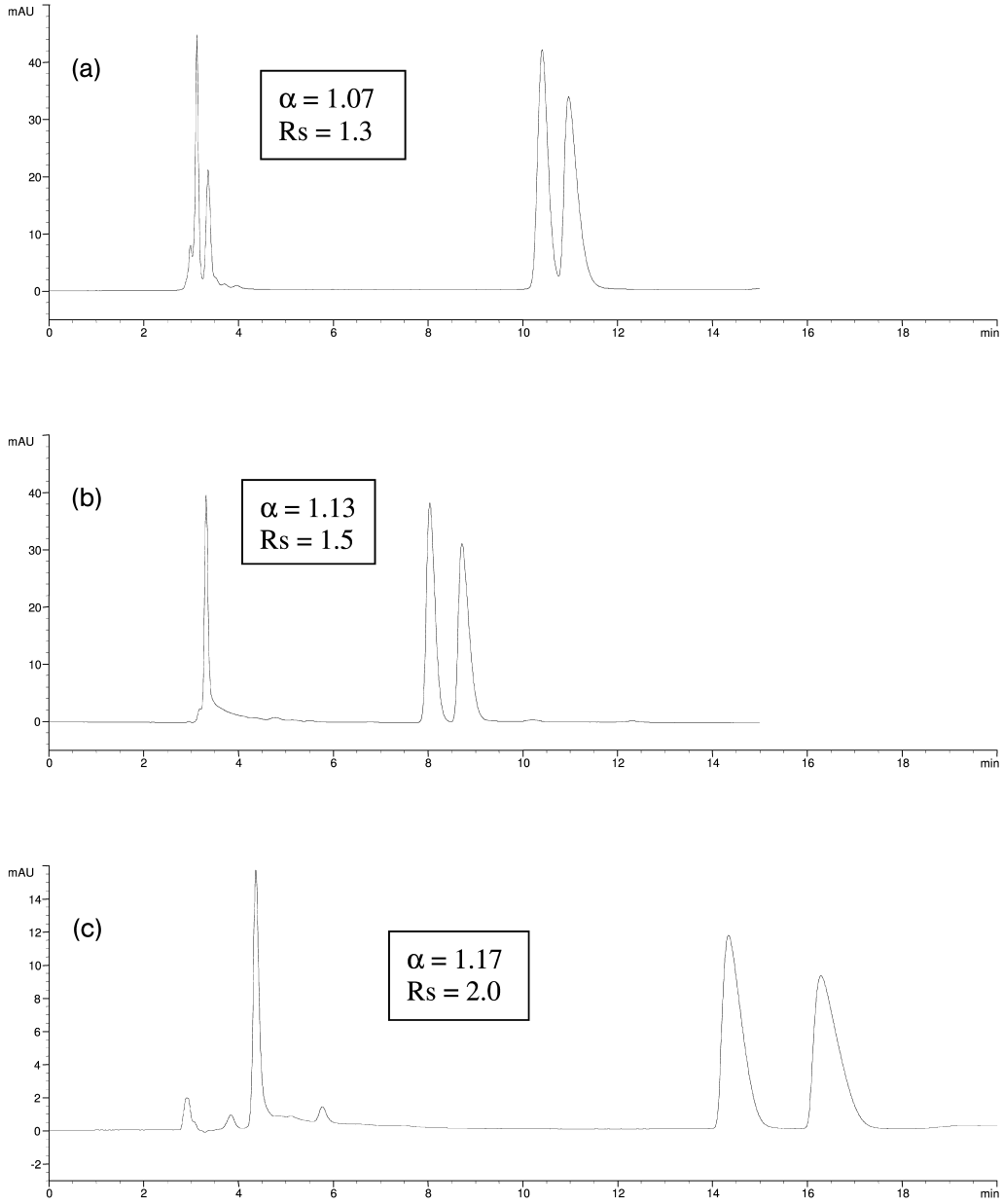


Fig. 1.4 From polar ionic mode to reversed-phase mode on a vancomycin (V2) column. Sample: α-methylbenzylamine. Mobile phase: (a) 100:0.05 MeOH–NH_4TFA; (b) as (a) + 25% H_2O; (c) as (a) + 50% H_2O. Flow-rate, 1 mL min^{-1}; UV detection at 254 nm.

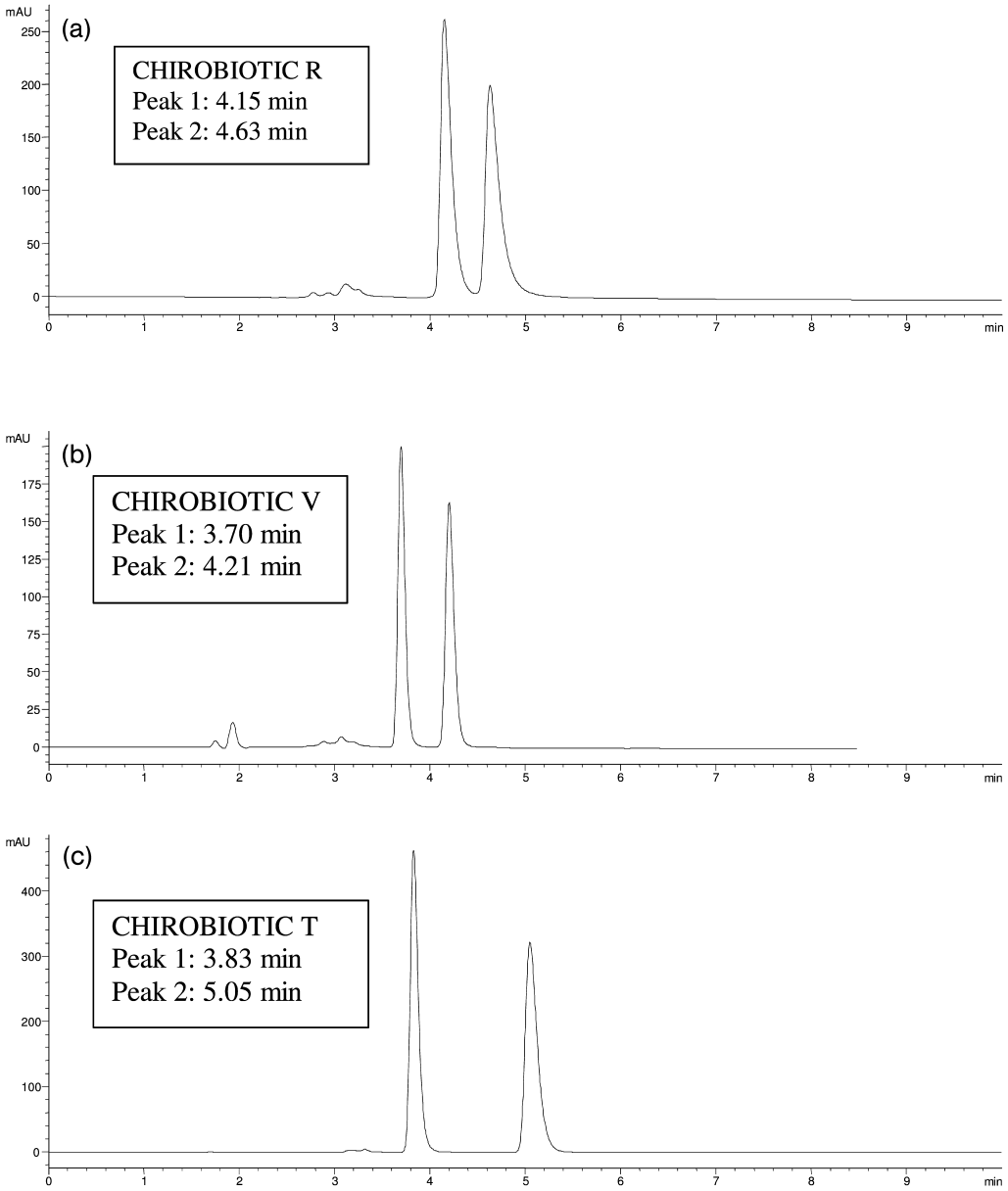


Fig. 1.5 Polar organic node separations on 5-methyl-5-phenyl-hydantoin using (a) ristcetin A, (b) vancomycin and (c) teicoplanin. Mobile phase, 100% MeOH; flow-rate, 1 mL min^{-1}; UV detection at 220 nm.

these systems, the eluent is composed of pure organic solvents with different degrees of polarity. For molecules with multiple H-bonding (>2) capability around the chiral center, the POM should be tried first. The best example is observed with 5-methyl-5-phenylhydantoin (Fig. 1.5). Pure MeOH or EtOH (or a combination of the two) yields very efficient separations. Other neutral compounds should be tried with typical normal phases such as the combinations of EtOH [or 2-propanol (IPA)] and hexane (or heptane). Again, the peptide chain of the macrocyclic glycopeptide CSPs provides ample opportunities for multiple H-bond interactions, aided by steric, π–π or dipole–dipole interactions to obtain effective chiral recognition. In addition the above-mentioned solvents, acetonitrile (ACN), tetrahydrofuran (THF), methylene chloride, methyl *tert*-butyl ether (MtBE) and dimethyl sulfoxide (DMSO) have been used as the major eluent component or as additives to control selectivity and the separation by modulating H-bond interactions, by reinforcing steric effects and/or by improving the compounds' solubility.

1.4 Complementary Effects

One of the unique characteristics of macrocyclic glycopeptide CSPs is the complementary effects among these six CSPs [18, 20]. Under the same mobile phase composition, if one CSP has shown marginal selectivity, other glycopeptide phases will most likely yield better selectivity. Also, by utilizing different linkers to the silica surface, enhanced selectivity could be obtained between vancomycin columns, V and V2. Teicoplanin demonstrated a similar effect between T and T2, for the same reason. Figure 1.6 demonstrates this complementary effect on these two phases. Also, propranolol, for example, is just baseline resolved on a teicoplanin column in the PIM. When the same mobile phase is used on a teicoplanin aglycone column, better separation is obtained. In addition, the elution order is reversed. Figure 1.7 demonstrates these unique phenomena. Also, as mentioned in the previous section, when one type of mobile phase did not yield satisfactory results, better separation may be obtained by switching to one of the other mobile phase types (see Fig. 1.4).

1.5 Method Development

The macrocyclic CSPs are multi-modal phases and can be switched from one mobile phase system to another without any deleterious effects. The PIM offers the advantages of broad selectivity, high efficiency, low back-pressure, short analysis time, extended column life, high capacity and excellent prospects for preparative-scale applications. Whenever a racemic compound is targeted for separation, its structure can give a hint as to which mobile phase/CSP combination

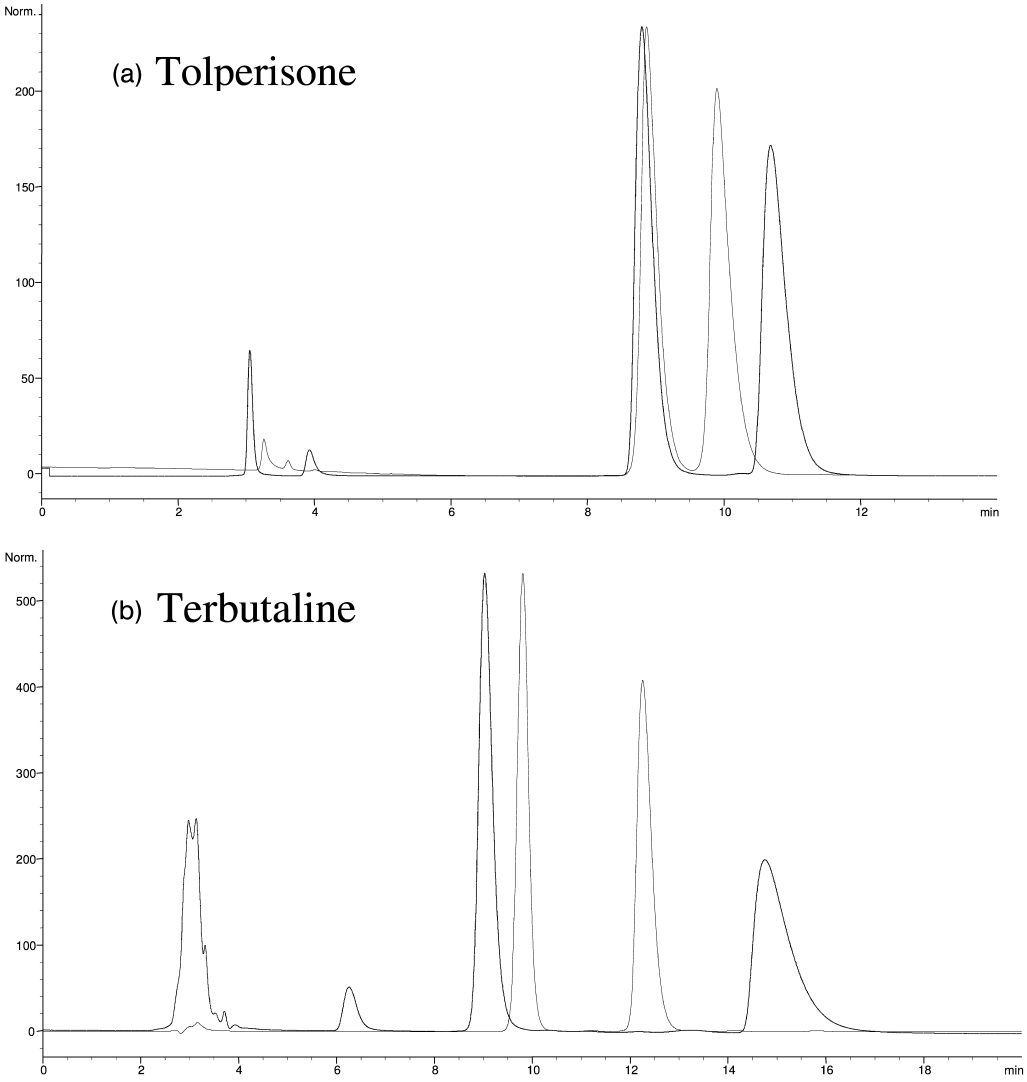


Fig. 1.6 Comparison of two vancomycin columns, V (solid line) and V2 (broken line), and two teicoplanin columns, T (solid line) and T2 (broken line) in polar ionic mode. (a) Tolperisone; (b) terbutaline. Mobile phase, 100:0.1 MeOH–NH_4TFA; flow-rate, 1 mL min^{-1}; UV detection at 230 nm.

should be approached. Table 1.4 summarizes the relationship between CSPs, mobile phase system and type of compound to be analyzed. A typical screening protocol in HPLC for the PIM is 100:0.1:0.1 (v/v/v) MeOH–HOAc–TEA whereas for the RP mode it is 20:80 MeOH–buffer (pH 5), for the POM it is 100% EtOH and for the NP mode it is 30:70, EtOH–heptane.

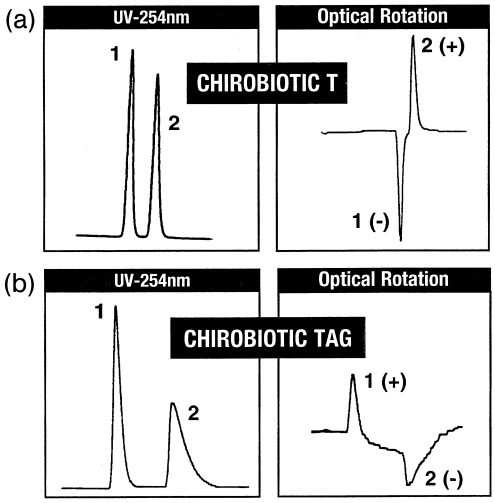


Fig. 1.7 Complementary effect and reversal of elution order on propranolol using (a) a teicoplanin and (b) a teicoplanin aglycone column. Mobile phase, 100:0.1 MeOH–NH_4TFA; flow-rate, 1 mL min^{-1}.

With LC/MS platforms, 100:0.5:0.3 (v/v/v) MeOH–HOAc–NH_4OH or 100:0.1 (v/w%). MeOH–ammonium formate is recommended for screening in the PIM. In the RP mode, volatile additives such as formic acid–acetic acid and ammonium acetate–formate salts can be used.

Table 1.4 Compound type versus mobile phase system on all the macrocyclic glycopeptide CSPs. A double tick means that the selection is preferred based on the statistics.

Mobile phase type [b]	Molecules [a]						
	Acidic (–)		Basic (+)		Neutral		
	PIM	RP	PIM	RP	POM	NP	RP
Vancomycin		√	√	√	√	√	√√
Vancomycin 2			√√	√√	√	√	√
Teicoplanin	√√	√√	√√	√ [c]	√	√	√√
Teicoplanin 2	√√	√√	√√	√ [c]	√	√	√
Teicoplanin aglycone	√√	√√	√		√√	√√	√
Ristocetin A	√√	√√			√	√	√√

a) Samples are classified into three groups according to their ionizable functionality around the chiral center.
b) PIM, polar ionic mode; RP, reversed-phase mode; POM, polar organic mode; NP, normal-phase mode.
c) Mobile phase consists of >70% ACN.

1.6
Optimization Procedures

1.6.1
Polar Ionic Mode

This anhydrous organic solvent system uses methanol as primary carrier with addition of small amounts of acid and base functioning as the primary mechanism to maintain proper charges on both the CSP and the ionizable compound being chromatographed. Since ionic interaction is the key, the ratio of acid to base controls both the selectivity and retention, because the changes in the ratio of acid to base affect the degree of charge on both the glycopeptides and the analytes. As in the case of the basic analyte mianserin (Fig. 1.8), the highest selectivity is obtained when the HOAc:TEA ratio is 3 : 1 whereas very little selectivity is observed when the ratio is 1 : 3. When the amino group is fully positively charged while the COOH of vancomycin maintains sufficient negative charge,

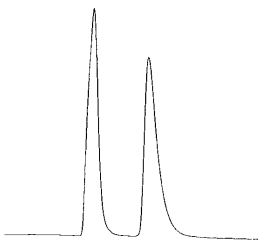
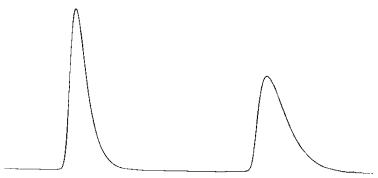
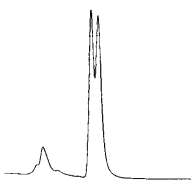
Example	Mianserin
Mobile Phase	MeOH/HOAc/TEA
100/0.1/0.1 Peak 1 – 6.21 min. Peak 2 – 7.36 min. Ratio: 1:1	
100/0.15/0.05 Peak 1 – 10.44 min. Peak 2 – 14.46 min. Ratio: 3:1	
100/0.05/0.15 Peak 1 – 3.43 Peak 2 – 3.58 Ratio: 1:3	

Fig. 1.8 Acid–base effect in the polar ionic mode on a vancomycin column.

(a) CHIROBIOTIC T2, 250x4.6mm
Sample: Atrolactic acid

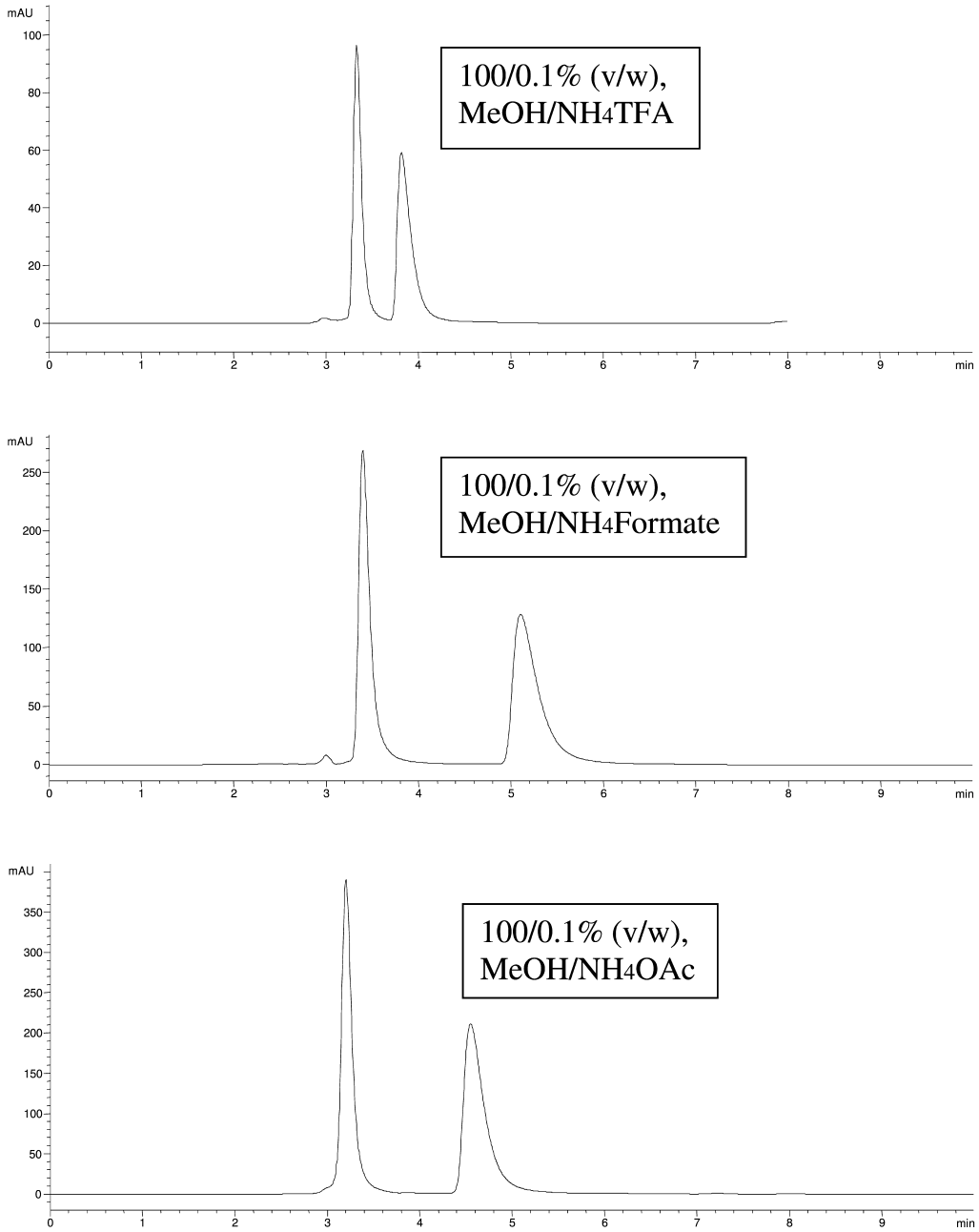


Fig. 1.9 Ammonium salt effect in the polar ionic mode on (a) a teicoplanin (T2)

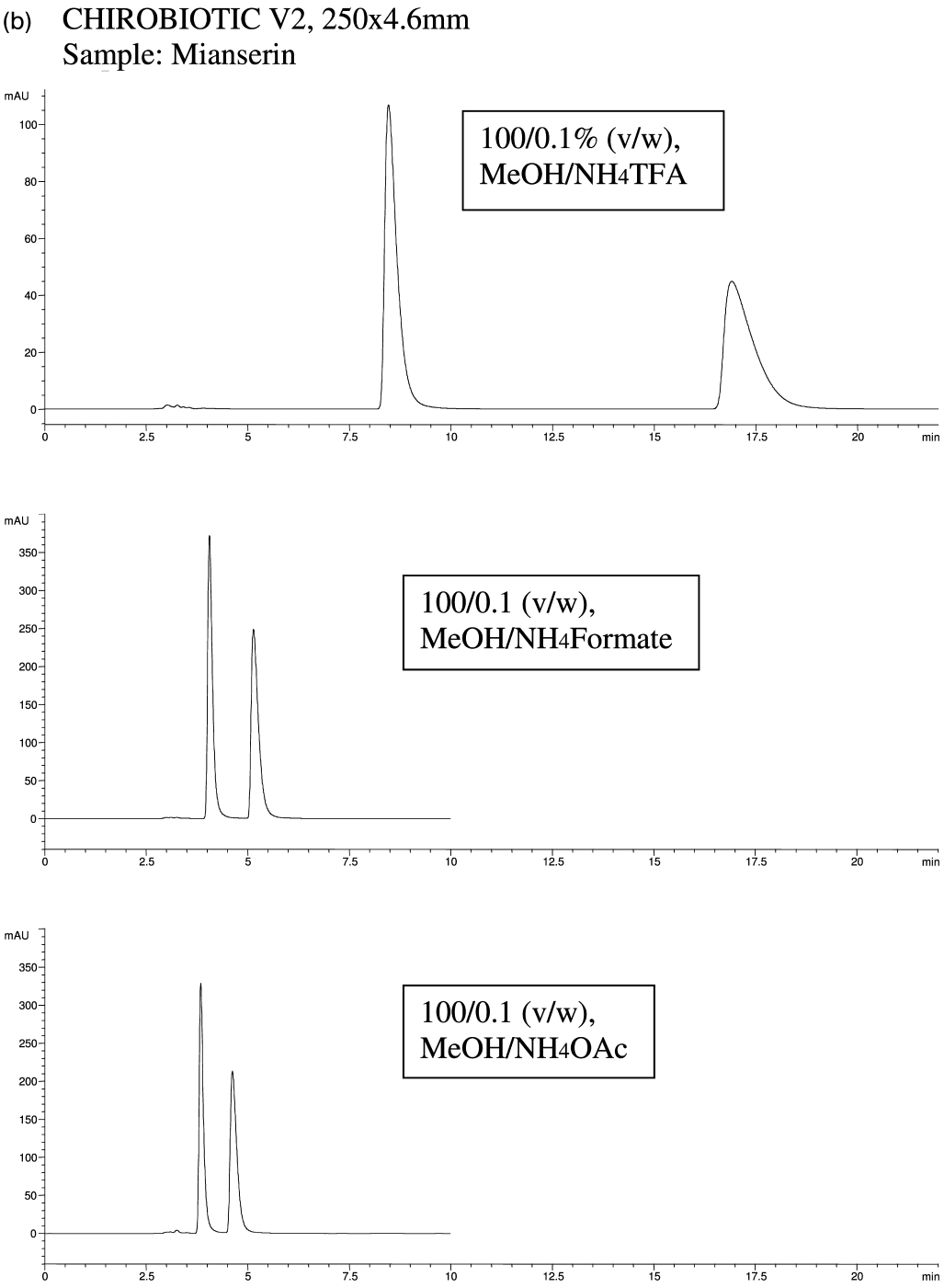


Fig. 1.9 (b) a vancomycin (V2) column. Flow-rate, 1 mL min^{-1}.

the selectivity is optimal. However, when the HOAc:TEA ratio is 1:3, the apparent pH exceeds the pK_a of this compound by two units. Thus, mianserin is in the free amine state so the ionic interaction is very weak (even though the COOH of vancomycin is fully negatively charged), leading to short retention and little selectivity. For acidic molecules, however, deprotonation of the acids is the key to better separation. Hence the best acid to base ratio (HOAc:TEA) is normally 1:2.

With LC/MS platforms, the HOAc:NH_4OH ratio could be 5:1 for basic molecules [24] whereas a 1:1 ratio favors acids. When ammonium salts are used in this system, ammonium trifluoroacetate favors basic molecules whereas ammonium acetate favors acidic molecules. Atrolactic acid with teicoplanin (T2) and mianserin with vancomycin (V2) are two typical examples of salt effects, shown in Fig. 1.9. This ionic interaction feature also favors detection by mass spectrometry as ionizable compounds need to be properly charged to obtain maximum sensitivity in an LC/MS system. Once the separation is established in HPLC, the method is easily transferred to LC/MS applications. In most cases, the retention factor is inversely proportional to the concentration of additives. The concentration range of these additives is typically 0.01–1.0%.

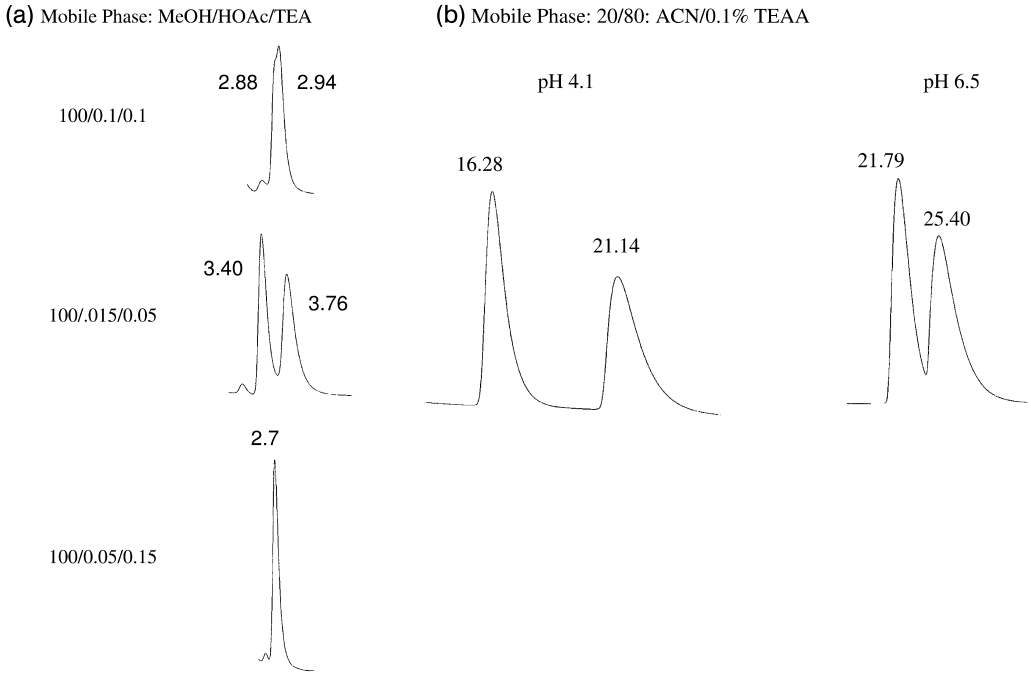


Fig. 1.10 Enantioseparation of mosapride on a vancomycin column. (a) Acid–base effect in polar ionic mode; (b) pH effect in reversed-phase mode.

1.6.2 Reversed-phase Mode

Retention and selectivity are controlled mostly by (1) the pH of the aqueous buffers and, to a lesser extent, concentration and (2) the type and concentration of organic modifiers.

1.6.2.1 pH Effects

Since ionic interaction is also a key mechanism in the RP system, the pH of the aqueous buffer has the greatest impact on the retention and, most importantly, the selectivity. When compounds were not separated well in other systems (PIM for ionizable compounds or POM/NP mode for neutral compounds), this system is the next logical step. Mosapride (base) is a typical example. It did not give satisfactory results in the PIM even with HOAc:TEA ratios up to 1:3. However, in the RP mode, a lower pH showed much better result, which was not unexpected. (Fig. 1.10). Again, the rule of thumb is that basic compounds

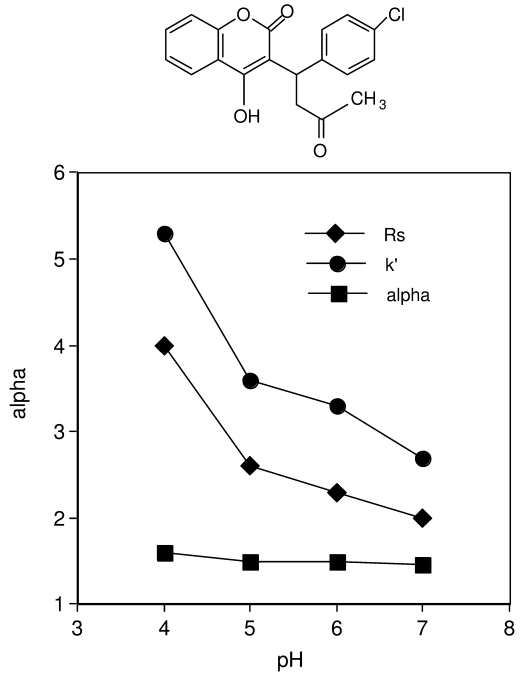


Fig. 1.11 Effect of pH on the retention, selectivity and resolution of coumachlor enantiomers on vancomycin CSP. Column, 250×4.6 mm i.d.; mobile phase, 10:90 acetonitrile–1% triethylammonium acetate; flow-rate, 1 mL min^{-1}; temperature, ambient.

favor lower pH buffers (around 3–4) whereas acidic compounds prefer higher pH buffers (6–7). It is within this pH range that both the analyte and the CSP are properly charged and the net interaction is the most effective. When dealing with neutral compounds, however, buffers with lower pHs usually showed better results, because a lower pH has the effect of suppressing the nonchiral retention mechanisms on a chiral stationary phase and to a lesser extent, the silanol group of silica gel, which in turn enhances the chiral interactions, leading to higher resolution. Coumachlor on vancomycin is a good example to demonstrate this (Fig. 1.11).

1.6.2.2 **Organic Modifier Effects**

Various organic modifiers can be used on the macrocyclic glycopeptide CSPs without any detrimental or memory effects. Common solvents such as MeOH, EtOH, IPA, ACN, THF, dioxane, methylene chloride, MtBE and DMF have been tested without any observable problems. The percentage of organic was found to follow a U-shaped relationship [25] with retention time (i.e. retentions are minimum at about 50% organic solvent) and the effects on retention and separation of buffer and pH values were shown to be dependent on the nature of the molecules. The impacts on these organic modifiers can be dramatic. The best example is given in Fig. 1.12 for the resolution of fluoxetine. Empirically,

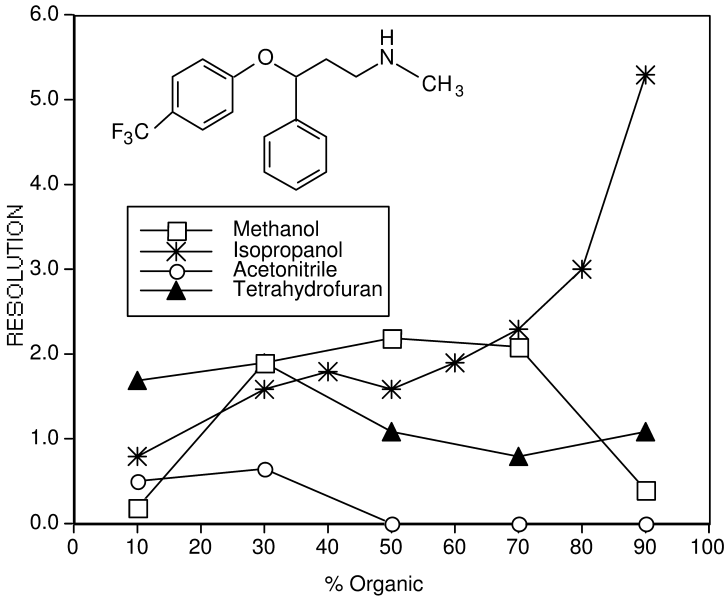
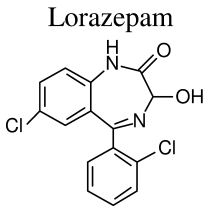


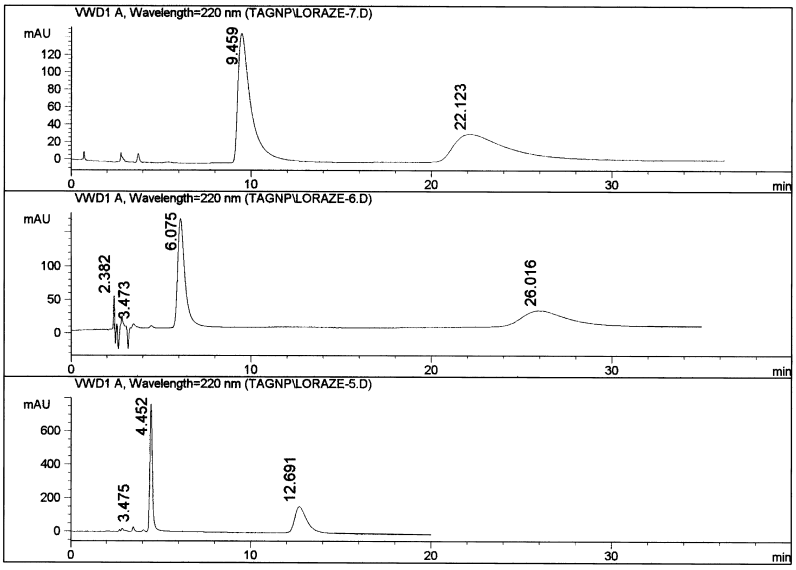
Fig. 1.12 Effect of organic modifiers on the resolution of fluoxetine enantiomers on vancomycin CSP. Column, 250×4.6 mm i.d.; flow-rate, 1 mL min^{-1}; temperature, ambient (23 °C). Courtesy of Scott Sharpe, Eli Lilly & Co.

THF and ACN work best on vancomycin whereas MeOH is good for teicoplanin and ristocetin A. Sometimes, the combination of any two solvents gives the best results.

1.6.3
Polar Organic/Normal-phase Mode

These mobile phase systems are suitable for neutral molecules only. The task for optimization is straightforward once selectivity is observed. Usually, the retention is controlled by the polarity of the solvent. Hence, if the retention time is short using MeOH or EtOH, then a mixture of EtOH (or IPA) and hexane (or heptane) should be tried. It has been reported that halogenated solvents, and also ACN, dioxane and MtBE or their combinations, have been used successfully on these CSPs. Figure 1.13 illustrates the example of lorazepam, separated on teicoplanin aglycone CSP, showing various solvent effects.





CH_3CN
1.0mL/min.
α=2.9

EtOH
1.0mL/min.
α=7.0

MeOH
1.0mL/min.
α=6.0

Fig. 1.13 Effect of alternative solvents on retention and resolution in the polar organic mode on teicoplanin aglycone.

1.6.4
Flow-rate and Temperature Effects

A general phenomenon observed on CSPs with inclusion cavities is that a decrease in flow-rate usually results in an increase in resolution. This unique feature has a significant impact in the RP mode (Fig. 1.14), because the mass transfer is slower in the process of inclusion complexation. Therefore, a lower flow-rate will enhance chiral recognition. Similar phenomena were observed in the PIM, especially when the retention factor was small (<1). However, flow-rate has little or no impact on the selectivity in the typical NP system.

Changes in temperature have some effects in all the mobile phase systems on these macrocyclic glycopeptide CSPs, because the binding constant between a solute and CSP involves several interactive mechanisms that change with temperature. The maximum operating temperature for these CSPs is 50 °C. Based on the linearity of van't Hoff plots, no conformation changes are observed between 5 and 45 °C [26–28] under the same mobile phase conditions. At higher

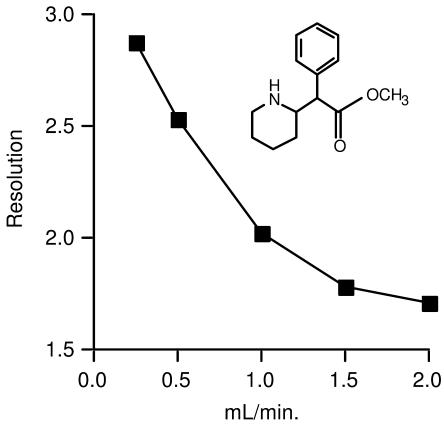


Fig. 1.14 Effect of flow-rate on the resolution of methylphenidate enantiomers on vancomycin CSP. Column, 250×4.6 mm i.d.; mobile phase, 95:5 methanol–1% triethylammonium acetate, pH 4.1; temperature, ambient (23 °C).

Table 1.5 Temperature effects on the chiral separation of *N*-carbamylphenylalanine using vancomycin CSP[a)].

Temperature (°C)	Retention factor (k'_1)	Selectivity (α)	Resolution (R_s)
0	0.51	1.39	1.5
5	0.39	1.34	1.3
15	0.38	1.23	1.0
22	0.31	1.20	0.8
35	0.27	1.11	0.7
45	0.22	1.00	0.0

a) Column, 250×4.6 mm i.d.; mobile phase, 10:90 acetonitrile–1% triethylammonium acetate, pH 4.1; flow-rate, 1 mL min^{-1}.

Table 1.6 Summary of optimization parameters on macrocyclic glycopeptide CSPs.

Polar ionic mode	1. Type of acid and base
	2. Acid:base ratio
	3. Concentration of acid and base
	4. Flow-rate
Reversed-phase mode	1. Type of organic modifier
	2. Concentration of organic modifier
	3. Type of aqueous buffer
	4. Concentration of aqueous buffer
	5. pH of aqueous buffer
	6. Flow-rate
	7. Temperature
Polar organic/normal-phase mode	1. Type of polar solvent
	2. Concentration of polar solvent
	3. Acid or base as modifier
	4. Temperature

temperatures, peak efficiency usually increases, at the expense of some decreases in enantioselectivity, in most cases. Inclusion complex formation is mostly diminished for most analytes in the temperature range 60–80 °C. However, lowering the temperature generally enhances the weaker binding forces, resulting in better chiral selectivity/separation (Table 1.5). The overall optimization parameters are summarized in Table 1.6 for all the mobile phase systems mentioned.

1.7 Amino Acid and Peptide Analysis

The enantiomeric separation of chiral underivatized natural and synthetic amino acids has been readily accomplished on these chiral stationary phases using simple alcohol–water- and alcohol–buffer-based mobile phases [29–35]. Successful separations have also been achieved for a wide range of *N*-blocked amino acids, such as *N*-FMOC-, *N*-acetyl- and *N*-*t*-BOC- [35–37] amino acids. The method development protocol and the optimization procedures are given in Table 1.7. Figure 1.15 shows the impressive separation of acidic amino acids, aspartic acid and glutamic acid, on a teicoplanin column. For LC/MS platforms, several publications on native amino acids [38, 39] and small peptides [38] using the electrospray ionization (ESI) mode and the atmospheric pressure chemical ionization (APCI) mode have also appeared.

In addition, single amino acid chiral/achiral isoforms in peptide sequences showed unique selectivity on macrocycle-based CSPs [35, 40]. Figure 1.16 shows the separation of six enkephalins on a teicoplanin (T2) column. This is a perfect example demonstrating the ability to separate single amino acid analogs (peaks

Table 1.7 Screening and optimization methods for natural and synthetic amino acids and *N*-blocked amino acids using teicoplanin-based CSPs (T, T2 and TAG).

	Amino acid type		
	Neutral	**Acidic**	**Basic**
Screening:			
Starting mobile phase	50:50 ACN–H_2O or 50:50 MeOH–H_2O	50:50:0.02 MeOH–H_2O–HCOOH	50:50 MeOH–20 mM NH_4OAc, pH 4.1
Optimization:			
Organic	% MeOH or ACN (ACN works best for lipophilic amino acids and peptides)	% MeOH or EtOH	% MeOH or EtOH
pH	No pH adjustment required	Test pH 2.8–7.0; best resolution is generally at lower pH	Test pH 2.8–7.0; best resolution is generally at lower pH
Buffer		Evaluate best buffer: NH_4TFA, NH_4OAc, NH_4COOH and its concentration	Evaluate best buffer: NH_4TFA, NH_4OAc, NH_4COOH and its concentration
Temperature (°C)	5–50	5–50	5–50
	***N*-Blocked amino acids**		
	***N*-FMOC**	***N*-*t*-BOC**	***N*-Acetyl**
Screening:			
Starting mobile phase	30:70 MeOH–20 mM NH4OAc, pH 4.1 (not TAG)	10:90 MeOH–buffer, pH 4.1 (not TAG)	30:70 MeOH–buffer, pH 4.1, or 100:0.1 MeOH–NH_4OAc

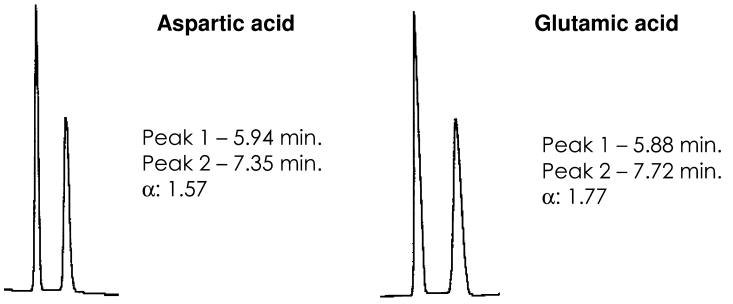


Fig. 1.15 Separation of bifunctional amino acids using a teicoplanin column. Mobile phase, 80:20:0.02 MeOH–H_2O–HCOOH; flow-rate, 0.8 mL min^{-1}; detection, ELSD.

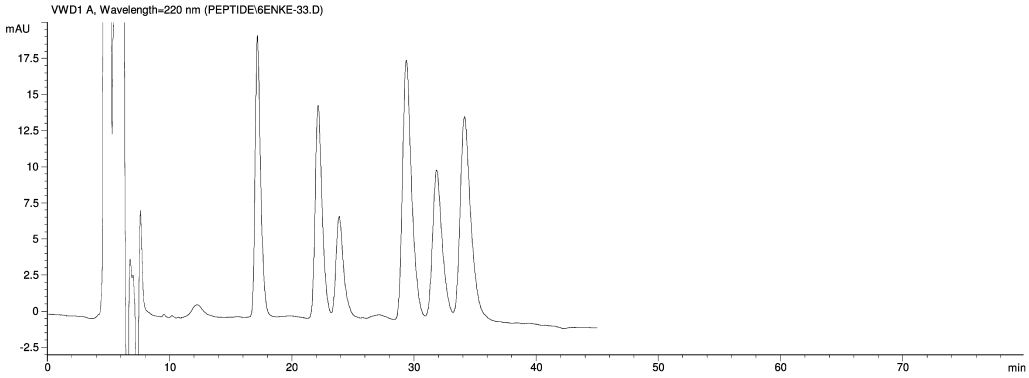


Fig. 1.16 Separation of six peptides of the enkephalin family (neutral peptides) using a teicoplanin column. Single amino acid analogs are peaks 2 and 3, peaks 4 and 5 and peaks 5 and 6. Chiral amino acid analogs are peaks 1 and 3 and peaks 3 and 6. Mobile phase, 75:25 acetonitrile–5 mM ammonium formate, pH 3.3; flow-rate, 0.5 mL min^{-1}; UV detection at 230 nm.

2 and 3, peaks 4 and 5 and peaks 5 and 6) and also single chiral amino acid isoforms (peaks 1 and 3 and peaks 3 and 6). In the latter cases, peptide analogs with D-amino acids eluted earlier than the L-form, which is the opposite of that noted for native amino acids and most of the *N*-blocked amino acids that have been reported. The mobile phases used were generally ACN–buffer-based systems that are LC/MS compatible, allowing higher sensitivity than UV detection. The percentage of organic was found to follow a U-shaped relationship with retention time (i.e. retentions are minimum at about 50% organic solvent) and the effects on retention and separation of buffer and pH values were shown to be dependent on the nature of the amino acids contained in the peptide. With traditional C_{18}, the sample load is often very limited, owing to mutual repulsion phenomena among the highly charged peptide molecules on the hydrophobic surface of the phase. The resulting peak shape problem is more severe when formic acid is used as an additive for potential LC/MS applications [41, 42]. Therefore, sample loading usually is limited to < 0.1 μg. The use of TFA as an additive can only alleviate some problems at the expense of losing sensitivity on ESI-MS platforms. However, for glycopeptide columns there is no such issue since these chiral stationary phases have inherent ionic characters such that charged peptide molecules will be associated and dissipated evenly with the CSPs. Figure 1.17 shows the chromatograms of three neurotensins (highly positively charged under the mobile phase conditions) separated on a teicoplanin (T2) column with different sample loadings. As can be seen, there were no changes in retention times and peak shapes from 0.14 to 2.0 μg injected for each peptide. Finally, a simplified method development and optimization protocol for peptide analysis is given in Table 1.8.

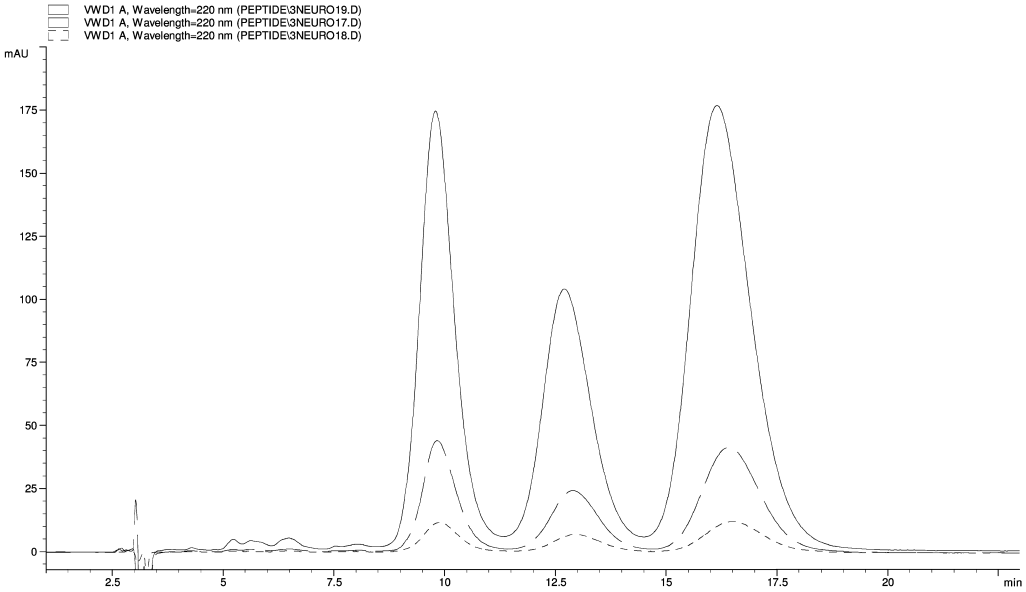


Fig. 1.17 Loading study of three angiotensin peptides on a teicoplanin (T2) column. Loading for each peptide: 0.14 μg (dotted line); 0.5 μg (broken line); 2.0 μg (solid line). Mobile phase, 20:80 acetonitrile–0.1% HCOOH; flow-rate, 0.8 mL min^{-1}; UV detection at 230 nm. No distortion of the peaks is observed.

Table 1.8 Method development protocol for peptide analysis using the teicoplanin-based CSPs (T, T2 and TAG). There are complementary effects between these CSPs also.

	Peptide type		
	Di-/tripeptide	**Bifunctional**	**Neutral**
Screening mobile phase:			
50% ACN with:	5 mM NH_4OAc, pH 4.1	0.1% HCOOH	5 mM NH_4 formate
Optimization:			
Organic	% ACN or a combination of ACN and MeOH	% ACN (U-shape effect)	% ACN (U-shape effect)
pH	2.8–6.8		2.8–6.2
Buffer concentration	2–50 mM	0.01–0.5%	2–50 mM
Temperature range (°C)	5–50	5–50	5–50
Flow rate (mL min^{-1})	0.5–2.0	0.5–2.0	0.5–2.0

1.8 Conclusion

The bonded macrocyclic glycopeptides vancomycin (Chirobiotic V and V2), teicoplanin (Chirobiotic T and T2), teicoplanin aglycone (Chirobiotic TAG) and ristocetin A (Chirobiotic R) have proven to be powerful chiral stationary phases for the separation of a wide variety of acidic, basic and neutral racemates. A unique variety of functional groups within the structures supports all the molecular interactions possible for chiral recognition. Bonding through several covalent linkages, these stable CSPs are multi-modal and can function in a variety of mobile phase conditions, both aqueous and nonaqueous, without memory effects or structural changes. In fact, the mechanisms of interaction are largely dictated by the type and composition of the mobile phase.

The most effective mobile phase condition is the polar ionic mode (PIM) for LC/MS and preparative applications. A number of examples have demonstrated the effects of steric interactions in the PIM and the benefits of utilizing the RP mode when dictated by the analyte's structure. The complementary nature of these phases is also a distinct advantage since it involves only the replacement of the column without further manipulation of the mobile phase conditions to obtain satisfactory results.

It was concluded in several publications that the addition of the Chirobiotic phases (three of them) to a screening protocol increased the enantioselectivity from a medium level of 87% to a high 96% [43]. Many of the overlapped separations afforded new opportunities for clinical applications and increased solubility for preparative applications.

Acknowledgments

The authors would like to thank Vicki Sutter and Michelle Roper for their assistance with the preparation of the manuscript.

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