Chiral Separation Techniques

A Practical Approach

Edited by
Ganapathy Subramanian

3rd, completely revised and updated edition

WILEY-VCH Verlag GmbH & Co. KGaA

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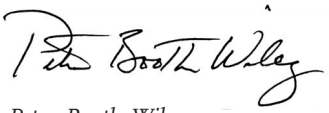

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Contents

Preface

The recognition of differences in the pharmacological activity of enantiomeric molecules has created the need to administer them as an isolated enantiomer. However, this problem of producing enantiopure products affects not only the pharmaceutical industry but other industrial sectors such as agrochemicals, food, petroleum and also biotechnology, all of which are increasingly concerned with developing techniques to produce the pure enantiomeric product. The majority of the chiral products are sold as racemates or mixtures. Today, with impending regulations on the production and use of chiral drugs, the need to monitor production, report the isomeric composition of products and study the pharmacological effects of drugs has resulted in more than 70% of the chiral chromatography market being accounted for by the pharmaceutical industry, with emphasis on both analytical- and preparative-scale processes.

The first commercially available stationary phase for chiral HPLC was introduced in 1981 and the continuous development of technology has resulted in several stationary phases being applied successfully in analytical and preparative separations of chiral molecules. The versatility of chiral stationary phases and their effective application in both analytical- and large-scale enantiopurification have been discussed in earlier books, *A Practical Approach to Chiral Separation by Liquid Chromatography* (ed. G. Subramanian, VCH, Weinheim, 1994) and *Chiral Separation Techniques: a Practical Approach* (ed. G. Subramanian, Wiley-VCH, Weinheim, 2001). The present book aims to bring to the forefront current developments in and successful application of chiral separation techniques, providing an insight for chemists, biochemists and chemical engineers, allowing a choice of methodology in the production of enantiopure substances of quality. For comprehensive overviews, the reader is referred to specialized review articles.

I am indebted to thirty-four authors and co-authors from laboratories from all over the world who have agreed to share their experience and knowledge.

Each chapter represents an overview of its chosen topic. Chapter 1 provides an overview of Method Development and Optimization of Enantioseparations Using Macrocyclic Glycopeptide Chiral Stationary Phases, while Chapter 2 provides an account of Role of Polysaccharides in Liquid Chromatography and Capillary Electrophoresis and Chapter 3 details the Analytical and Preparative Potential of Immobilized Polysaccharide-derived Chiral Stationary Phases. Chapter 4 gives an account of Supercritical Fluid Chromatography in Chiral Separations

while Ligand Exchanges in Chiral Separation are detailed in Chapter 5. SMB Technology and Its Application are discussed in Chapters 6 and 7. Chiral Crown Ethers are detailed in Chapter 8 and Chapter 9 surveys the separation of Amino Acids and Hydroxy Acids. Capillary Electrophoresis is discussed in Chapter 10. Countercurrent Chromatography and Molecular Imprinting in Chiral Separations are detailed in Chapters 11 and 12. Chapter 13 gives an outline of Biosensors in Enantioselectivity and CEC and MEKC Coupled to Mass Spectrometry in the Analysis of Chiral Products are discussed in Chapters 14 and 15. Chapter 16 describes the Application of the Chiral Polarimeter in Enantioseparations. An insight into Preparative Chromatography in Drug Discovery is detailed in Chapter 17.

This book should be helpful to pharmaceutical chemists, biochemists, molecular biologists, pharmacologists and scientists in the agrochemical, food and biotechnology fields.

I wish to express my sincere thanks to Dr. Frank Weinreich for inviting me to edit this volume and Dr. Romy Kirsten and her colleagues in the publishing department for their sustained enthusiasm and support through the production of this book.

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

Thomas E. Beesley and J. T. Lee

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-

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.

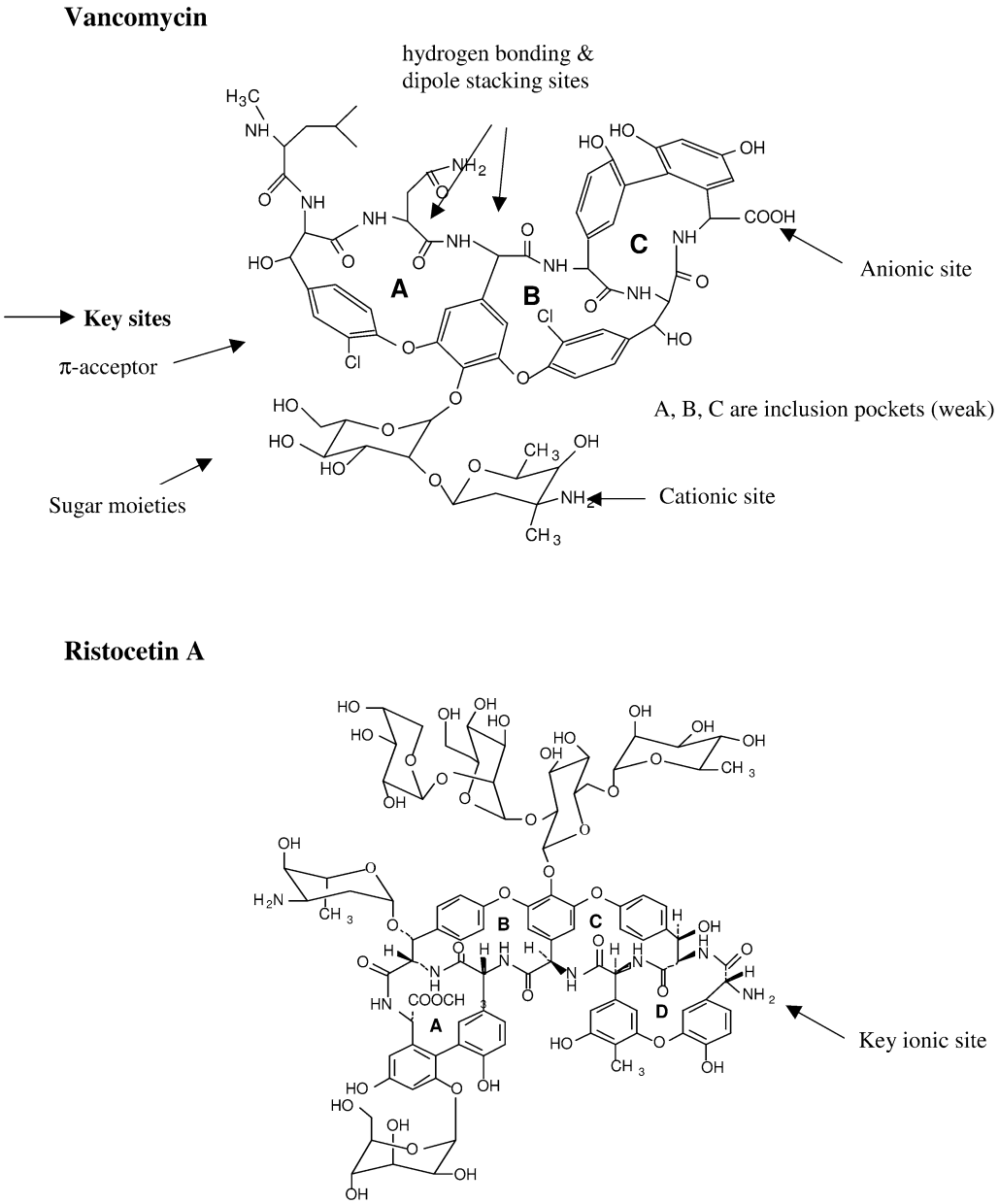
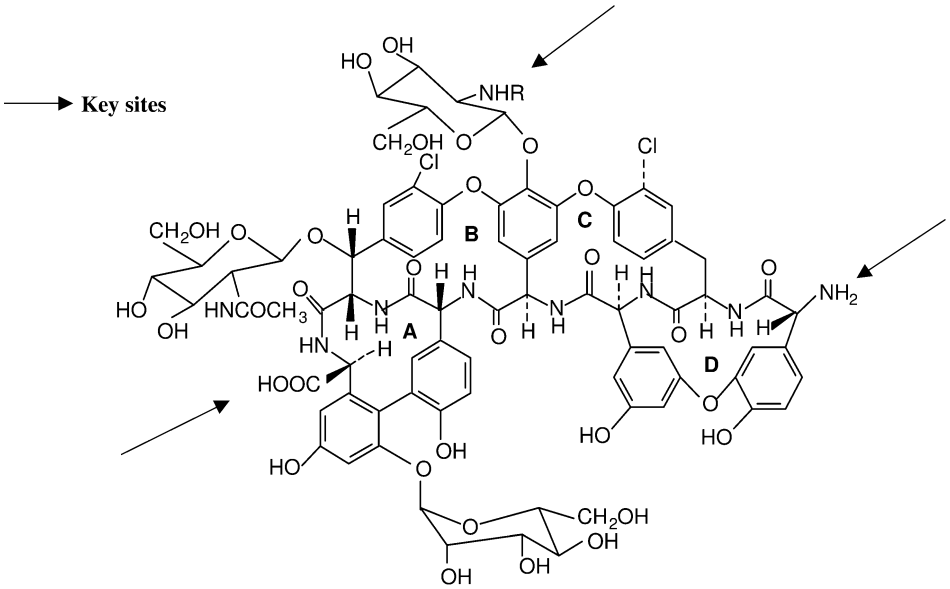


Fig. 1.1 Proposed structures of glycopeptide CSPs.

Teicoplanin



Teicoplanin Aglycone

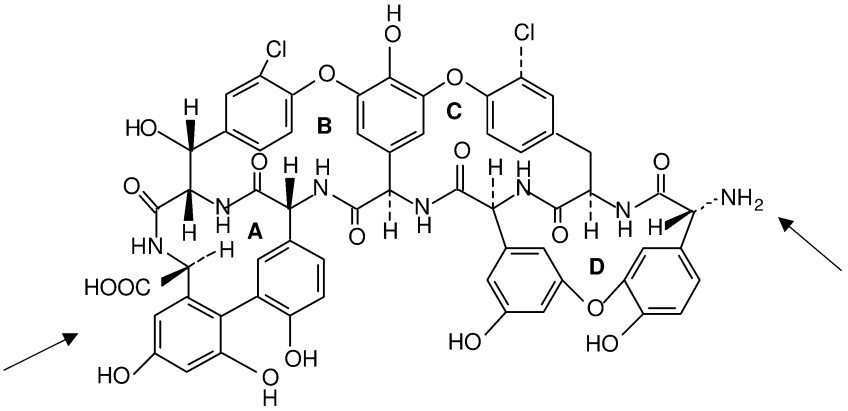


Fig. 1.1 (continued)

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

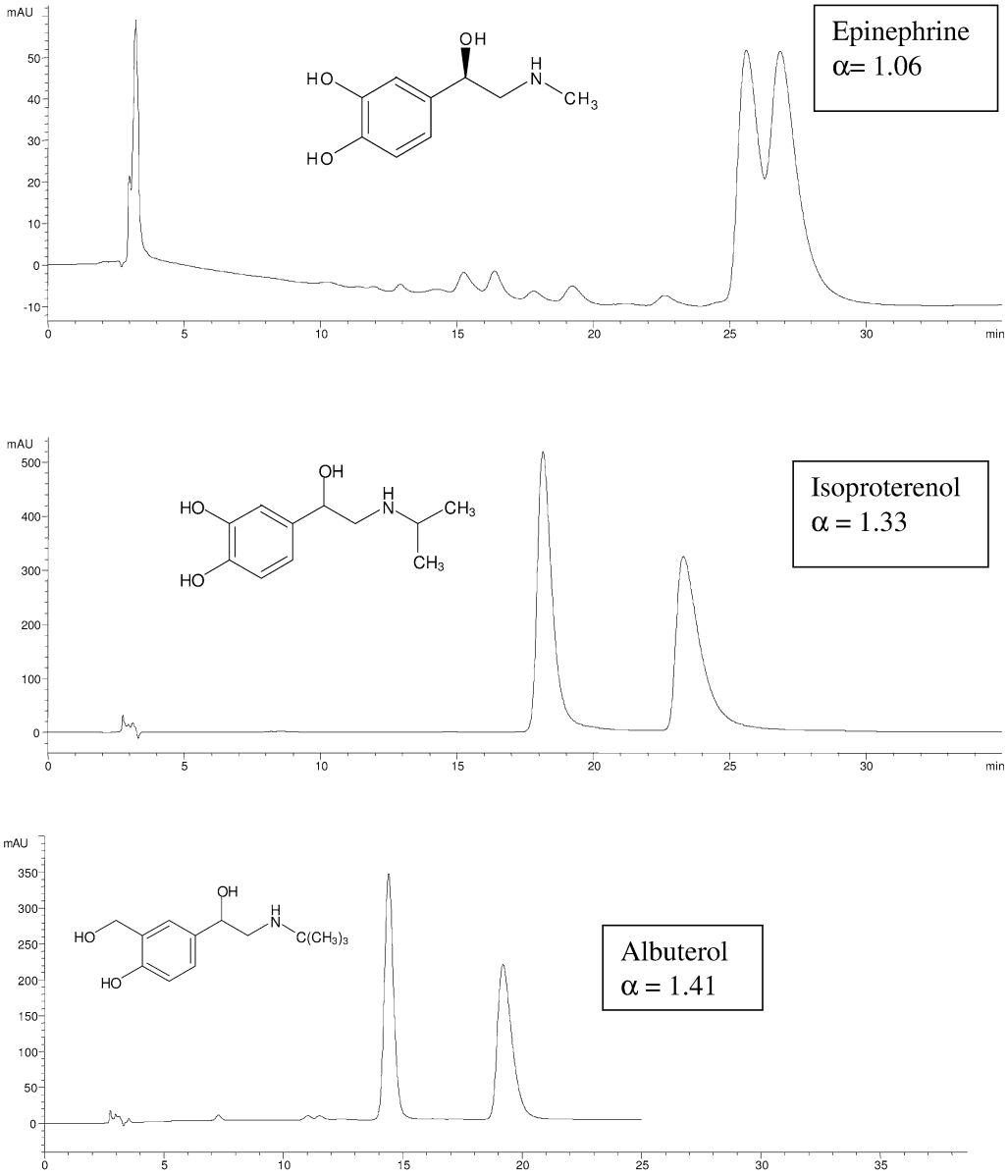


Fig. 1.2 Selectivity comparison for structurally related amino alcohols using a teicoplanin column in the polar ionic mode. Mobile phase, 100:0.1:0.1 MeOH–HOAc–TEA; flow-rate, 1 mL min^{-1}; UV detection at 230 nm.

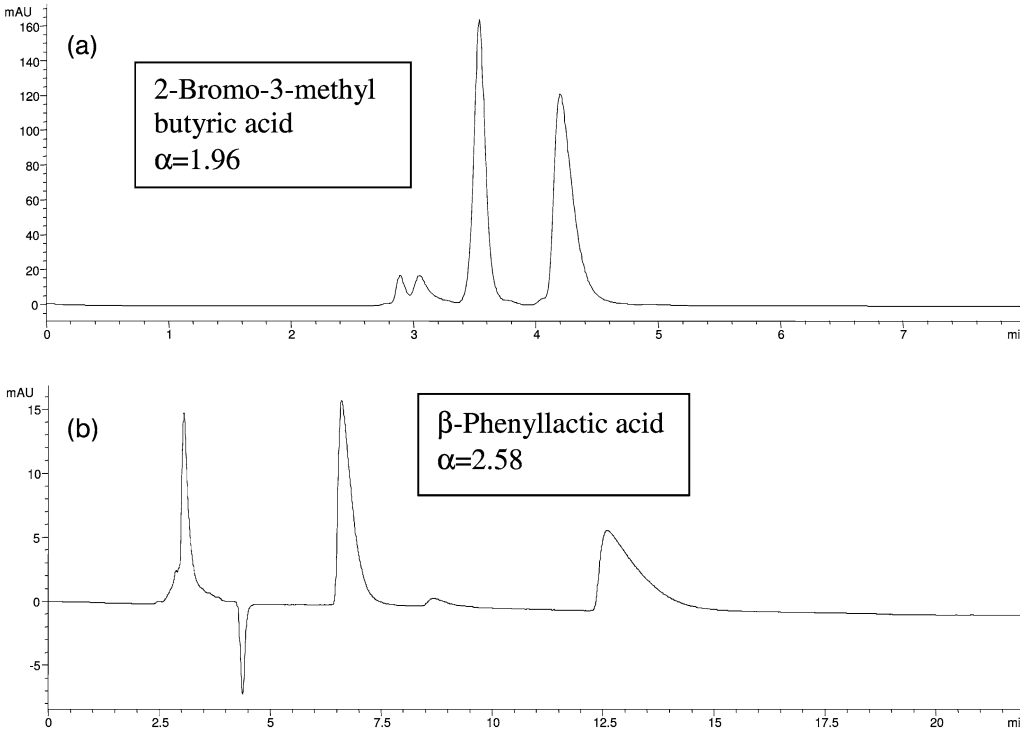


Fig. 1.3 Enantiomeric separation of *α*-hydroxy-/halogenated acids on ristocetin CSP. Column, 250×4.6 mm i.d.; mobile phase, 100:0.1 MeOH–NH_4OH; flow-rate, 1 mL min^{-1}; UV detection at 230 nm. (a) 2-Bromo-3-methylbutyric acid; (b) *β*-phenyllactic acid.

(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

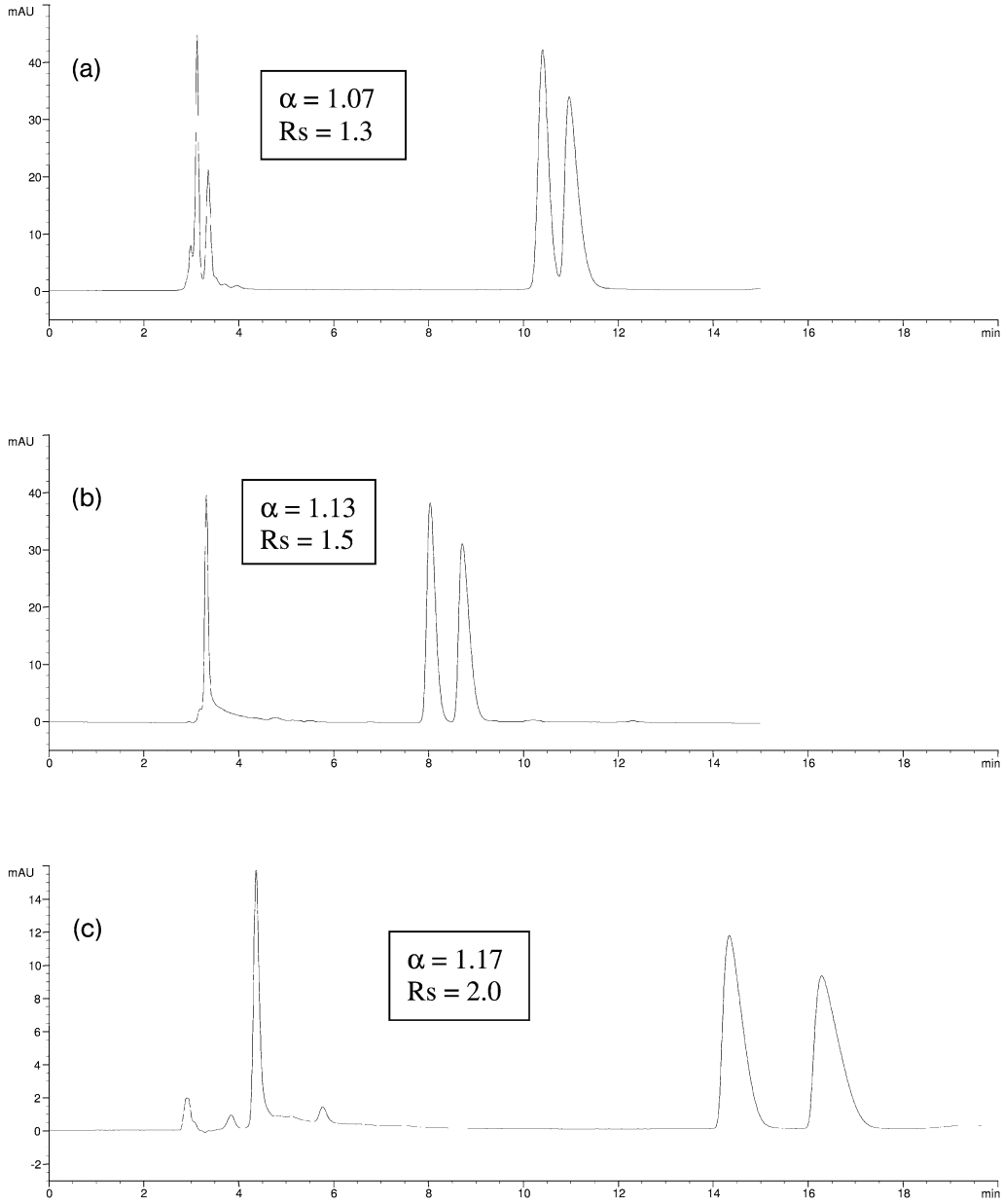


Fig. 1.4 From polar ionic mode to reversed-phase mode on a vancomycin (V2) column. Sample: α-methylbenzylamine. Mobile phase: (a) 100:0.05 MeOH–NH_4TFA; (b) as (a) + 25% H_2O; (c) as (a) + 50% H_2O. Flow-rate, 1 mL min^{-1}; UV detection at 254 nm.

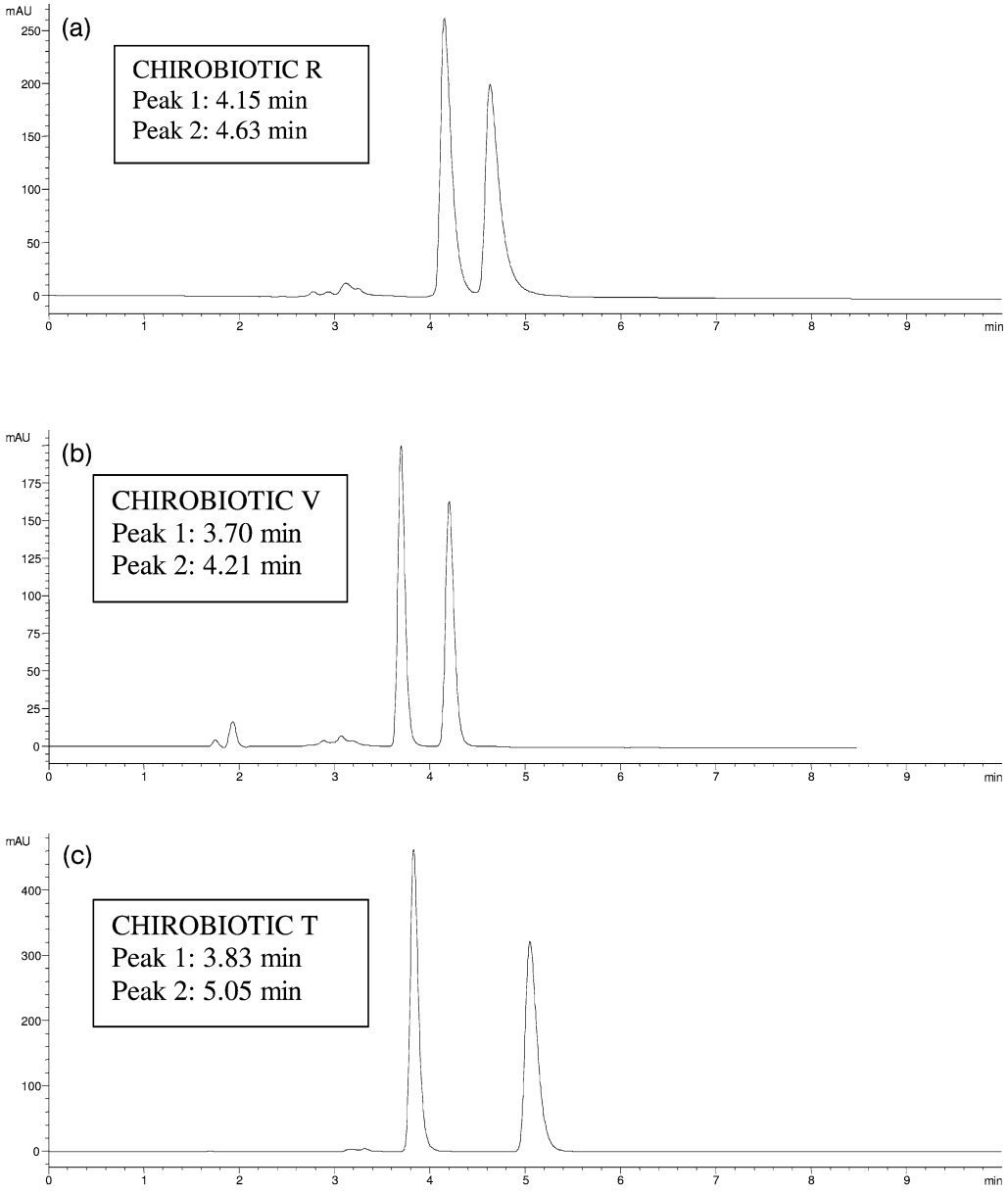


Fig. 1.5 Polar organic node separations on 5-methyl-5-phenyl-hydantoin using (a) ristcetin A, (b) vancomycin and (c) teicoplanin. Mobile phase, 100% MeOH; flow-rate, 1 mL min^{-1}; UV detection at 220 nm.

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

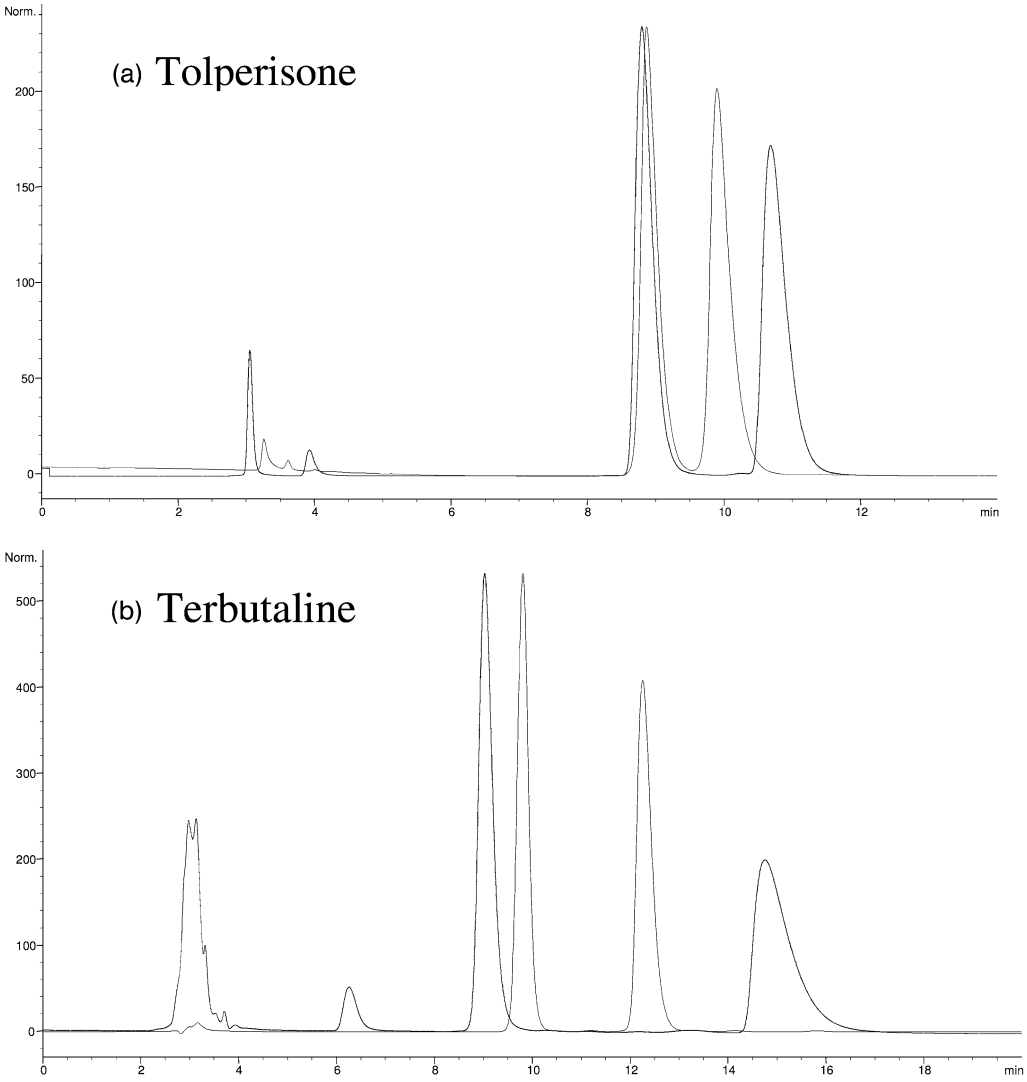


Fig. 1.6 Comparison of two vancomycin columns, V (solid line) and V2 (broken line), and two teicoplanin columns, T (solid line) and T2 (broken line) in polar ionic mode. (a) Tolperisone; (b) terbutaline. Mobile phase, 100:0.1 MeOH–NH_4TFA; flow-rate, 1 mL min^{-1}; UV detection at 230 nm.

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.

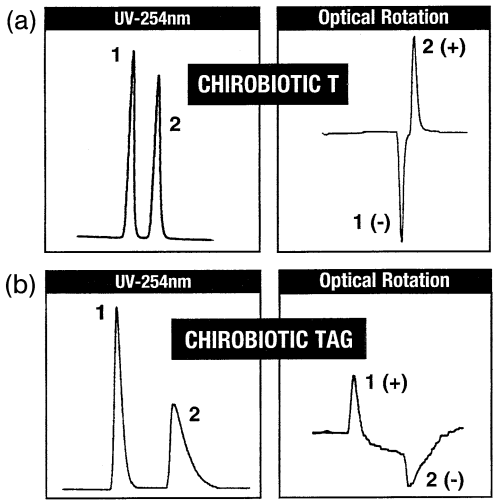


Fig. 1.7 Complementary effect and reversal of elution order on propranolol using (a) a teicoplanin and (b) a teicoplanin aglycone column. Mobile phase, 100:0.1 MeOH–NH_4TFA; flow-rate, 1 mL min^{-1}.

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,

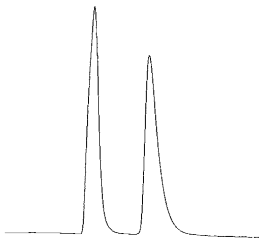
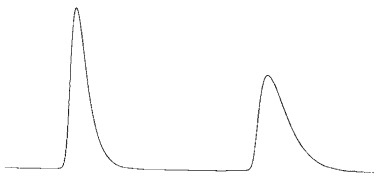
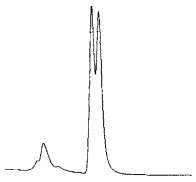
Example	Mianserin
Mobile Phase	MeOH/HOAc/TEA
100/0.1/0.1 Peak 1 – 6.21 min. Peak 2 – 7.36 min. Ratio: 1:1	
100/0.15/0.05 Peak 1 – 10.44 min. Peak 2 – 14.46 min. Ratio: 3:1	
100/0.05/0.15 Peak 1 – 3.43 Peak 2 – 3.58 Ratio: 1:3	

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

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

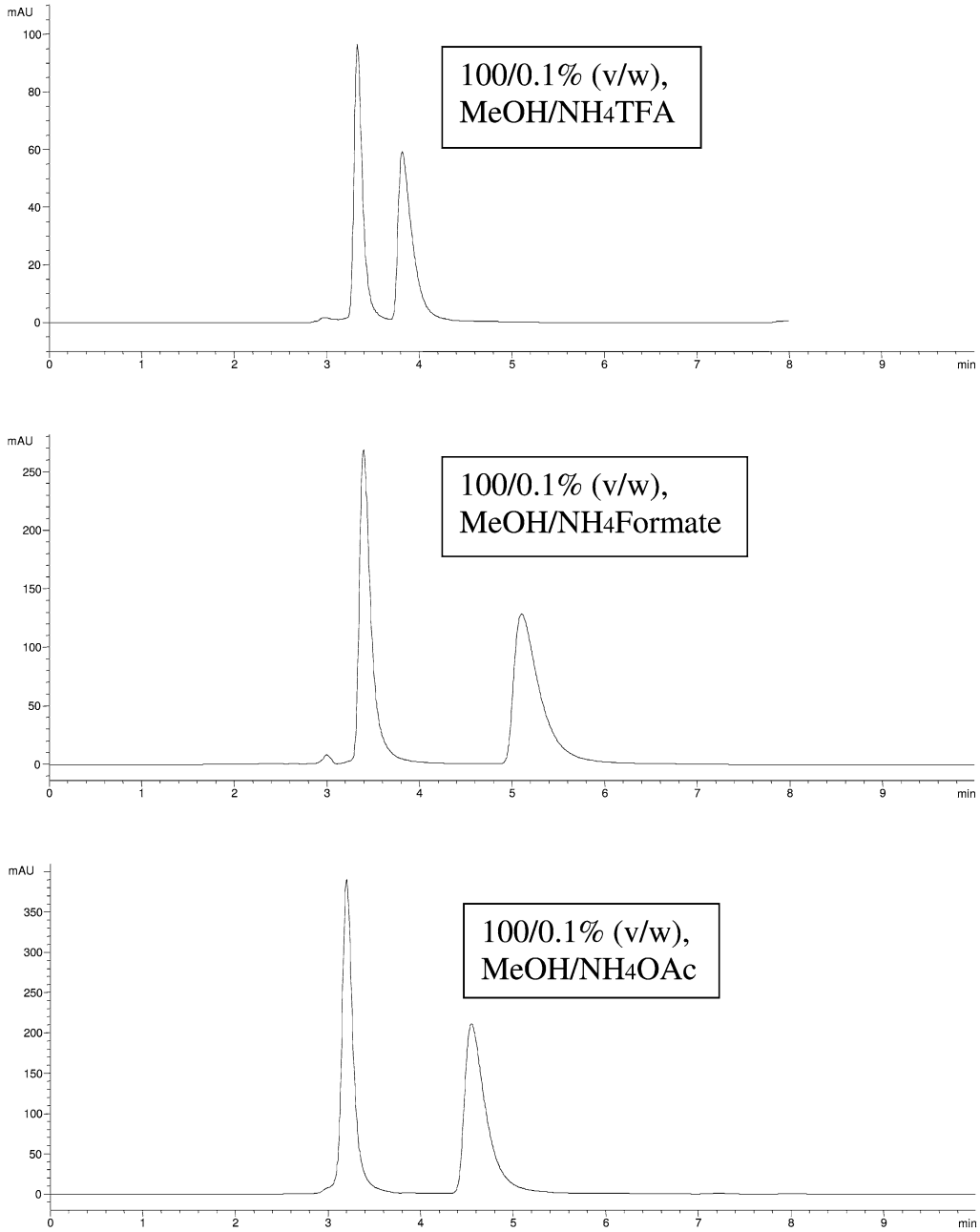


Fig. 1.9 Ammonium salt effect in the polar ionic mode on (a) a teicoplanin (T2)