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Review

Enantioselectivity in capillary electrophoresis using the macrocyclic antibiotics

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Abstract

The macrocyclic antibiotics recently have been shown to exhibit powerful enantioselectivity towards numerous compounds. There are a number of ways that one can alter enantioselectivity in the macrocyclic antibiotic-based separation schemes. The macrocyclic antibiotics are enantioselective for positively-charged solutes using the ansamycins and enantioselective for anionic compounds using the glycopeptides. Within a given class of antibiotics such as the glycopeptides, enantioselectivity may also be altered by use of micelles, uncoated vs. coated capillaries, or manipulation of operating parameters such as pH or organic modifiers. In this work, we will examine the various ways to alter enantioselectivity in the macrocyclic-based separations. © 1997 Elsevier Science B.V.

Keywords: Chiral selectors; Reviews; Enantiomer separation; Enantioselectivity; Antibiotics; Ristocetin A; Teicoplanin; Vancomycin; Rifamycins; Profens; Amino acids; Carboxylic acids

Contents

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1. Introduction

Capillary electrophoresis (CE) is a powerful analytical separation technique that brings together speed, quantification, reproducibility, and automation to the inherently high-resolving methods of electrophoresis [1–3]. In the past few years the use of CE has grown rapidly, and many new applications have been introduced into this field of analytical separation [3–7]. Since the principle of separation for CE and other methods such as HPLC differ, CE provides an excellent complementary technique [8]. Another advantage is the ease in changing separation media in the capillary column. In terms of method development, this unique property of CE enables one to quickly and effectively alter the run buffer to screen various separation media at a minimum cost, with small amounts of reagents consumed.

Most CE methods for the separation of chiral compounds have their origins in HPLC [3,8]. There are two major categories of enantioseparation: (1) techniques that use a chiral additive in the run buffer and (2) techniques that involve a chiral stationary phase immobilized either on the capillary wall or on an appropriate support [9–11]. Although the use of wall-immobilized chiral stationary phases are feasible, the addition of a chiral selector to the free solution is the most common technique [12–27]. So far the most widely used and successful additives for CE enantiomeric separations are cyclodextrins and their derivatives [28–31]. This is due to the fact that cyclodextrins are of an optimal size to form inclusion complexes with a significant number of chiral compounds. Another contributing factor to their widespread use is the ability to derivatize the secondary hydroxyls at the rim of the cyclodextrin with various functional groups, which can improve solubility and provide unique selectivity for separations [28–35]. In spite of their popularity, the need for more diverse and potentially more powerful chiral selectors still remains an intensive area of interest.

Recently, Armstrong and co-workers first demonstrated macrocyclic antibiotics as a broad, useful new class of chiral selectors in CE, HPLC and TLC to separate a wide variety of enantiomers [36–43]. There are hundreds of racemic solutes that have been electrophoretically resolved via macrocyclic antibiotics chiral selectors, including numerous non-steroidal anti-inflammatory compounds, antineoplastics, hydroxy acids, lactic acids, herbicides, rodenticides, α-amino alcohols, and most *N*-blocked amino acids.

2. The macrocyclic antibiotics

Few new chiral selectors have had as immediate and dramatic an impact as have the macrocyclic antibiotics [3,8,36–46]. These compounds contain multiple stereogenic centers and a variety of functional groups. Much of the macrocyclic antibiotics' selectivity can be attributed to their ability to form multiple interactions, e.g., hydrogen-bonding groups, hydrophobic pockets, aromatic groups, amide linkages, etc. One of the primary interactions of the macrocyclic antibiotics are thought to be charge-to-charge or ionic interactions [37–39,43]. Other interactions are hydrogen bonding, steric repulsion, hydrophobic, dipole–dipole, and π–π interactions. The presence of the various functional groups is also attributive to the degrees of ionization and solubility of the macrocyclic antibiotics. Most macrocyclic antibiotics are ionizable (they can be used in either charged or uncharged states) and are sufficiently soluble in aqueous buffers and solvents such as those used in CE. In addition, the macrocyclic antibiotics are structurally diverse and are complementary in the types of compounds they can resolve. (Principle of complementary separations are discussed in a later section). The structural types of the macrocyclic antibiotics include compounds such as macrocyclic polyene–polyvols, macrocyclic glycopeptides, ansa compounds and peptides. However, the macrocyclic glycopeptides, in particular, have been shown to exhibit superior enantioselectivity in comparison to the other classes of the macrocyclic antibiotics.

2.1. Structural properties

Despite their common properties, macrocyclic antibiotics differ in several of their physico-chemical properties and often exhibit different selectivities towards many compounds [8,37,43,46]. The glycopeptide antibiotics ristocetin A, teicoplanin, and vancomycin, consist of an aglycon portion of fused macrocyclic rings, which form a characteristic 'basket' shape, various pendant carbohydrate moieties,

and numerous functional groups as shown in Fig. 1. They differ structurally in a number of ways. The aglycons of vancomycin and teicoplanin contain 2-chloro-substituted aromatic rings, while the analogous portion of ristocetin A has no chloro substituents. Of the three macrocyclic antibiotics, vancomycin with 18 stereogenic centers, 3 macrocyclic rings, and an attached disaccharide D-glucose and vancosamide, is the smallest. Ristocetin A with 38 stereogenic centers and 3 fused macrocyclic rings is larger than teicoplanin, which has 23 stereogenic centers. All three of the macrocyclic glycopeptides ristocetin A, vancomycin and teicoplanin possess a number of ionizable groups, which affect their charge and chiral recognition properties. While all three compounds contain an amine on the aglycon portion and amino saccharide moieties, only teicoplanin has two amino saccharides, both of which are N-acetylated (Fig. 1). Both ristocetin A and teicoplanin have primary amine groups on their aglycon portions, whereas vancomycin contains a secondary amine. Moreover, the aglycon portions of both vancomycin and teicoplanin have a carboxylic acid moiety, whereas in ristocetin A this group is esterified. All other ionizable groups on these compounds are the phenolic moieties. These structural

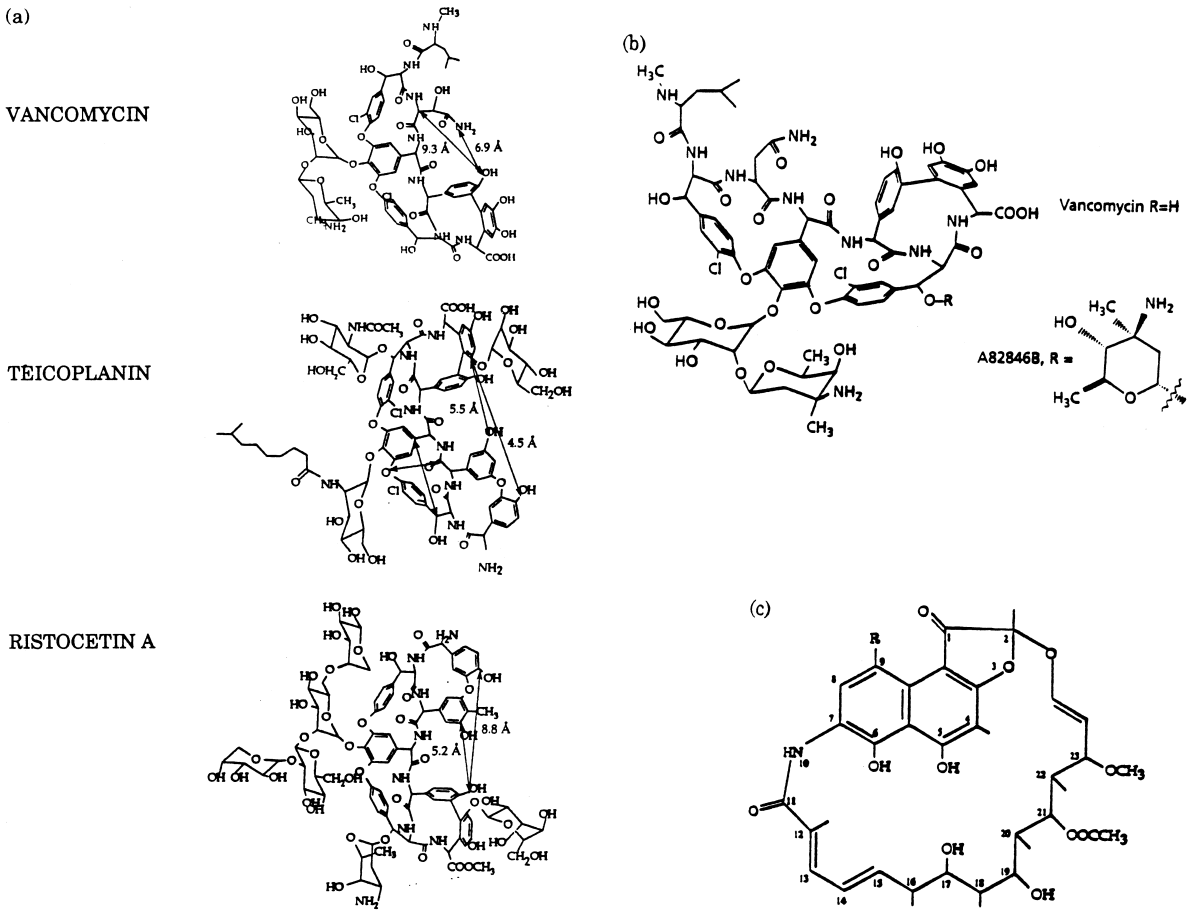


Fig. 1. (a) Structures of the glycopeptide antibiotics, vancomycin, teicoplanin and ristocetin A, showing the similarities and differences in their structures (Reprinted with permission from [43], 1966, American Chemical Society); (b) Structures of the glycopeptide vancomycin and its analogue, A82846B, which differs from vancomycin in that it contains an additional epivancosamine and has an epimeric disacchride amino sugar. (Reprinted with permission from Ref. [46], 1996 Advanstar Publication); (c) Structures of rifamycin B (R=–OCH_2COOH) and rifamycin SV (R=–OH). (Reprinted with permission from Ref. [44], 1995, Elsevier).

differences provide a means for altering the selectivity when performing chiral separations.

The ansamycins, rifamycin B and rifamycin SV, on the other hand, have a characteristic ansa structure, which include a ring structure or chromophore spanned by an aliphatic chain. All rifamycins have a highly substituted aliphatic bridge, and differ from one another in the type and location of the substituents on their naphthohydroquinone ring [38,44]. Specifically, rifamycin B differs from rifamycin SV by an R group attached to the naphthohydroquinone ring at position 9 as indicated in Fig. 1. Rifamycin SV has a hydroxyl (-OH) R group, whereas rifamycin B has an oxyacetic acid (-OCH_2COOH) R group. Table 1 lists a number of physico-chemical properties of ristocetin A, teicoplanin, vancomycin and rifamycin B and SV as well as their approximate costs.

2.2. UV–Vis spectral properties

Some macrocyclic antibiotics absorb quite weakly in the UV and visible spectrum while others possess large conjugated groups and absorb intensely. As one might predict from the aromatic rings in the structures, the glycopeptides ristocetin A, teicoplanin and vancomycin absorb strongly in the UV spectral region, and have similar spectra as shown in Fig. 2 [39,41,43]. In acidic solutions, each glycopeptide macrocyclic antibiotics absorbs strongly at the wavelength below 250 nm and exhibits a minimum at approximately 260 nm. In alkaline solutions, however, the minima is lost at 260 nm, and there is a general shift in the spectra to slightly longer wavelengths with increasing pH for ristocetin A and teicoplanin spectra, but less for vancomycin [39,41,43]. It is believed that this is due to ionization of the phenolic groups and/or partial decomposition of the macrocyclic antibiotics [43]. Despite strong absorbance in the UV–Vis region, direct detection of

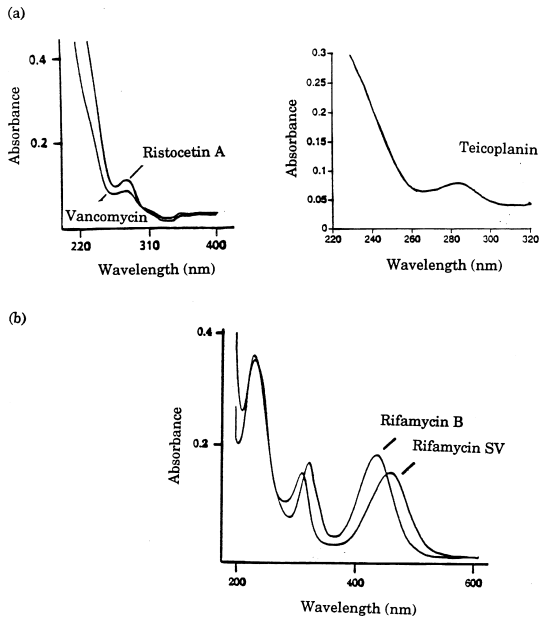


Fig. 2. UV–Vis spectra of (a) vancomycin, ristocetin A and teicoplanin in acidic solutions, showing minima at 260 nm; (b) UV–Vis spectra of rifamycin B and SV at pH 7.

Table 1
Physico-chemical properties of the ansamycins, rifamycins B and SV and glycopeptide antibiotics, ristocetin A, teicoplanin and vancomycin

	Ansamycins		Glycopeptides		
	Rifamycin B	Rifamycin SV	Ristocetin A	Teicoplanin	Vancomycin
Solute types	positive	negative	negative	negative	negative
M_r	755	698	2066	1877	1485
Stereogenic centers	9	9	38	23	18
Hydroxyls	4	5	21	15	9
Aromatic rings	2	2	7	7	5
pH	wide range	wide range	5–7	4–7	3–7
O.M. effect	significant	significant	significant	minimal	minimal
Stability	1 wk	1 wk	4 wks	2–3 wks	1 wk
Costs/gram	$32	$41	$2300	N/A in U.S.	$99
Buffer conc.	25 m*M*	25m*M*	1–5 m*M*	1–5 m*M*	1–5 m*M*

most solutes is nevertheless possible since the effective concentrations normally used in the run buffer is usually 1–5 m*M*. As for the ansamycins, both rifamycin B and SV absorb strongly in the UV and visible spectral regions due to their naphthohydroquinone ring. Each compound exhibits maxima at approximately 220, 304 and 425 nm and minima at 275 and 350 nm. Interestingly, changes in solution pH between 4.5 and 9.5 result in relatively small changes in the absorbance spectrum [38,44]. This is undoubtedly due to the lack of functional groups, which results in little changes in ring conjugation when pH changes. Because of their strong absorbance and concentration range used (20–25 m*M*) in the running buffer, most rifamycins are monitored via indirect detection. This type of detection generally results in negative peaks (a reduction in absorbance from a high background signal), and therefore places a limitation on the chiral selector concentration in the run buffer. When concentrations as high as 30 m*M* rifamycin B or SV are used, the background absorbance is excessive, resulting in unacceptable signal-to-noise ratios [38].

2.3. Electrophoretic properties

As discussed previously, the glycopeptide antibiotics contain a number of ionizable group whose charge is governed by pH. At operational pH values of 3.5–7.5, these groups are usually protonated and serve as sites for hydrogen bonding. Fig. 3 shows how the electrophoretic mobilities of vancomycin and ristocetin A vary as a function of pH. At pH 7.5 ristocetin A exhibits a zero electrophoretic mobility in 0.1 *M* phosphate buffer, which is slightly higher than that found in vancomycin. Therefore, at pH less than 7.5, ristocetin A, like vancomycin, has an overall positive charge and interacts electrostatically with anionic compounds. Vancomycin has six reported pK_a values: 2.9, 7.2, 8.6, 9.6, 10.5 and 11.7 [39]. It is believed that the last three pK_a values are from the phenolic groups. Vancomycin exhibits an effective electrophoretic mobility at pH 7.2 in 0.1 *M* phosphate buffer. Thus, at pH ranges of 4–7, such as those commonly employed in CE separations, both ristocetin A and vancomycin have an overall positive charge. Teicoplanin does not begin to possess an overall positive charge until the pH value approaches

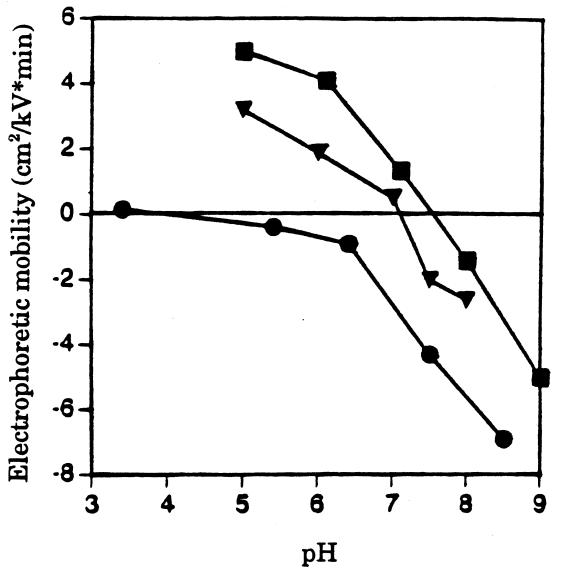


Fig. 3. Plot showing the effect of pH on the electrophoretic mobility of ristocetin A (■), vancomycin (▲), and teicoplanin (●) in 0.1 *M* phosphate buffer. (Reprinted with permission from Ref. [43], 1996 American Chemical Society).

pH of 4.5. Clearly the electrophoretic mobility versus pH curve and the p*I* of teicoplanin are different from those of the other two glycopeptides as shown in Fig. 3. At least two factors can account for the observed difference in the shape of electrophoretic mobility vs. pH curve of teicoplanin [43]. First, it is believed that due to the lack of an additional amine on the saccharide moieties (both aminosaccharides on teicoplanin are *N*-acylated), the main ionizable groups in teicoplanin are the single carboxylate and primary amine on the aglycon portion. Hence, at pH values above 3.8, teicoplanin carries a slight negative charge and migrates in the opposite direction to vancomycin and ristocetin A (against the EOF) [43]. Second, it is believed that teicoplanin self-associates to form an aggregate, which may affect its solution properties [43,47]. This is a property the compound shares in common with rifamycin B and SV, both of which exhibit aggregation properties. The ansamycin rifamycin B is a dibasic compound, hence, its electrophoretic mobility is opposite to the direction of the electroosmotic flow [38]. In fact, the electrophoretic mobility of rifamycin B in a solution of 2-propanol–0.1 *M* phosphate buffer (pH 7) was found to be -3.2 cm^2 min kV [38].

2.4. Chemical stabilities

The macrocyclic glycopeptide antibiotics are soluble in water, buffers and acidic aqueous solutions and less soluble at neutral pH [8,39,41,43]. They are moderately soluble in polar aprotic solvents such as dimethylformamide and dimethylsulfoxide and are relatively insoluble in most organic solvents. One factor to consider in selecting a macrocyclic antibiotic for use in CE is the stability of the compound in aqueous and in hydroorganic solvents. All of the glycopeptide macrocyclic selectors decompose with time under conditions commonly used in CE. Upon degradation, the migration times of the test solutes usually increase [39,41]. Vancomycin has been shown to be the least stable macrocyclic antibiotic. Solutions of vancomycin within the pH range of 5–7 deteriorate within 6–7 days at 4°C [43,45]. Teicoplanin solutions in the same pH range are somewhat more stable. As evidenced in the EOF velocities and enantioresolutions, these values have been shown to be reproducible for approximately 2–3 weeks. Upon degradation, it is not uncommon for the residence time of the analytes to increase and resolutions to decrease. The most hydrolytically stable macrocyclic antibiotic appears to be ristocetin A in solution, which can be used up to 4 weeks when certain precautions are taken [43]. The glycopeptide solutions should be refrigerated at 4°C overnight or when not in use. Under high temperature (≥35°C) as well as pH outside 4–7 range, the stabilities of the glycopeptide antibiotics can be greatly reduced. For example, stabilities of vancomycin, teicoplanin and ristocetin A solutions at room temperature (22°C) are reduced to 2–3 days, 3–4 days and 6–7 days, respectively. Upon heating the glycopeptide antibiotics under the pH range of 7–12 and at slightly elevated temperature (above 22°C), complete deterioration can be observed [43]. It is believed that under such conditions the macrocyclic rings are opened via hydrolysis of the amide bonds as well as possible cleavage of the saccharide moieties from the ring structures. Such deterioration is evidenced in a slight yellow discoloration of the solutions. Eventually, a murky solution forms, and the glycopeptide degradation products may form precipitates in solution [39,43]. The deterioration can cause a significant increase in baseline noise, as well as a reduction in separation performance. Interestingly, while it is not yet known whether this is coincidental, the stability of the glycopeptide antibiotics in aqueous solution increases with increasing number of attached saccharide moieties.

Solid glycopeptide antibiotics may be stored up to 2 months under hydrated conditions at 4°C. However, the most effective way to store the antibiotics is under anhydrous conditions and at <0°C [39,43]. It is of importance to note that semi-degraded glycopeptide antibiotics can sometimes produce acceptable enantioseparations. For example, when a resolution of 10 is obtained with a 'fresh' antibiotic solution, a semi-degraded mixture may still provide a more than adequate baseline resolution between 2 and 5. However, generally when semi-degraded glycopeptide antibiotics are employed as chiral selectors, there is an increase in migration time and baseline noise, as well as a decrease in enantioresolution.

The ansamycin rifamycin B is slightly soluble in water. The solubility increases in the short chain alcohols and acetone. Rifamycin B and SV form stable yellow to orange solutions depending in part on pH and organic cosolvents [38,44]. As the compounds begin to degrade in solution they turn dark red and eventually begin to precipitate from solution as degradation continues.

3. Separations using the macrocyclic antibiotics

3.1. CE separations in uncoated capillaries

3.1.1. The glycopeptides — vancomycin, ristocetin A, and teicoplanin

CE may be performed in either coated or uncoated columns which provides another means to alter the selectivity for a specific analysis. We will first begin by examining macrocyclic antibiotic-based separations performed in uncoated capillaries and examine the underlying mechanism of separation. Using either ristocetin A, teicoplanin or vancomycin, in the run buffer in CE with uncoated fused-silica capillary column, numerous racemates, including N-blocked amino acids, non-steroidal anti-inflammatory drugs, and antineoplastic compounds have been resolved [36,39,41–43,45]. The N-derivatized amino acids include highly fluorescent moieties as 6-amino-

quinolyl-N-hydroxysuccinimidyl carbamate (AQC), dansyl, or PHTH amino acids. The strong UV chromophores such as N-3,5-dinitrobenzoyl (DNB) and N-3,5-dinitropyridyl (DNP_{yr}) amino acids, as well as simple blocking groups, e.g., N-benzolyl, N-acetyl or N-formyl amino acids have also been resolved. For many of these compounds, the enantioselectivity of the chiral selectors is substantial. Resolution values between 10 and 20 are not uncommon, with most of the enantiomeric separations having resolutions greater than 2. Table 2 lists some of these separations using the macrocyclic antibiotics as the chiral selectors in an uncoated capillary. Fig. 4a shows the separation of dansylglutamic acid in 2 m*M* vancomycin in phosphate buffer. Ristocetin A also has been successfully used as a chiral selector in CE to resolve more than 120 racemic compounds [41]. By use of dilute solutions of ristocetin A a number of carboxylic-acid-containing compounds not effectively separated by the other glycopeptides have been separated. These compounds include mandelic acid and several of its derivatives, β-phenyllactic acid, tropic acid, and 2-bromo-3-methylbutyric acid.

The similarities in structure and action, which relate the glycopeptides vancomycin, ristocetin A, and teicoplanin to one another, extend to their enantioselectivities as well [43,46]. In most cases, the glycopeptide antibiotics exhibit highest enantioselectivities for compounds containing an acidic or anionic moiety such as carboxylate, phosphate, and sulfonate groups. Armstrong and co-workers [43] have demonstrated that when these groups are either α or β to the stereogenic center there seems to be an enhancement in enantiorecognition, particularly when a chiral compound contains a carbonyl group, an aromatic ring, or an amide nitrogen in the α, β or γ position to the stereogenic center. The glycopeptide amine groups are believed to be one of the primary sites of interaction, where the initial contact of an analyte to the glycopeptides occur [43]. In the study of vancomycin–Cu^{2+} complex it has been shown that when the secondary amine group is not available to interact with the analyte, effective enantioselectivity is not observed [48]. This has been demonstrated by using copper–vancomycin complexes where the secondary amine group is preferentially bound by copper. Poor to no enantioselectivity is observed when this secondary amine is bound to copper, even though the primary amine is free and available to interact electrostatically. This strongly indicates that the secondary amine plays a pivotal role in enantiorecognition with anionic solutes. Conversely, the carboxylic groups on the chiral selector do not appear to have a significant role in enantiorecognition under the conditions employed.

The glycopeptide antibiotics are complementary to one another in their enantioselectivities. By changing the glycopeptide antibiotics used as the chiral selectors, we can often substantially change the enantiomeric selectivity of the system. Armstrong and coworkers proposed that if only a partial enantioresolution can be obtained with one glycopeptide, there is a high probability that a baseline or better separation may be obtained with another glycopeptide [43]. This indicates that their basic enantioselective retention mechanism are related. While it is not yet possible to predict which glycopeptide antibiotic will give the greatest resolution for a given analyte, it appears that resolution may be improved by using one of the structurally related macrocyclic antibiotics. Of the 28 compounds studied by Armstrong and co-workers [43], only the antineoplastic compounds, methotrexate, and a few derivatized amino acids were baseline separated with all three glycopeptides. The most versatile glycopeptide antibiotics seem to be ristocetin A, followed by vancomycin, and teicoplanin, respectively. However, there are specific examples where only one or another macrocyclic antibiotic can achieve a separation. For example, the worse enantioresolution of fenoprofen is obtained when ristocetin A is used as a chiral selector (R_s = 0.9) [43]. Under the same conditions, when the chiral selector is changed to teicoplanin, R_s increases to 1.1 and the best enantioselectivity is observed with vancomycin, R_s increasing to 3.0. Ristocetin A appears to yield the greatest enantioresolutions for the aromatic derivatized amino acids, i.e., AQC, FMOC, N-benzoyl, dansyl, PHTH and 2,4-DNP, while vancomycin gives better resolution for the aliphatic derivatized amino acids (N-formyl and N-acetyl). In general, ristocetin A can resolve about as many racemates in CE as vancomycin and teicoplanin combined [39–41,45,46].

Enantioselectivity may also be altered by either modifying the solute or chiral selector. The slight

Table 2
Enantiomeric resolutions, migration times and apparent mobilities of racemates separated with ristocetin A, teicoplanin and vancomycin in uncoated and coated capillaries and with SDS–vancomycin buffers in 0.1 *M* phosphate buffer

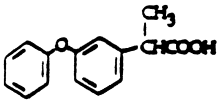
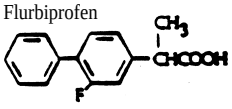
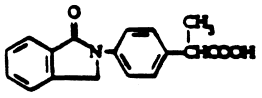
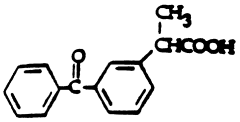
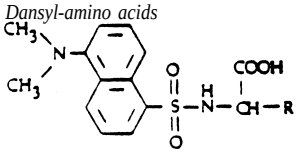
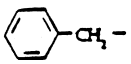
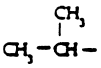
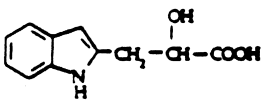
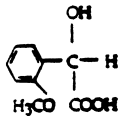
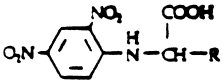
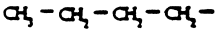
Compound	R_s	Time(1)[a]	Time(2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]	pH/m*M*/C.S.
Nonsteriodal anti-inflammatory drugs						
Fenprofen	0.9	16.1	16.5	−7.6	−7.8	6/2(R)[d]
	1.2	18.2	18.7	10.4	10.1	6/2(R)[e]
	1.1	16.3	16.7	−9.1	−9.4	6/2(T)[f]
	3.6	33.3	38.1	−8.7	−9.3	7/5(V)[g]
	1.6	17.6	18.5	7.5	7.1	6/2(V)[h]
Flurbiprofen	1.7	27.2	28.1	−8.8	−9.0	6/2(R)[d]
	8.2	39.4	52.4	−8.0	−9.1	7/5(V)[g]
	2.4	19.4	21.0	6.8	6.2	6/2(V)[h]
	0.9	19.0	19.2	−11.7	−11.8	7/2(V)[i]
Indoprofen	1.3	12.7	13.1	−4.8	−5.2	6/2(R)[d]
	2.3	19.6	20.6	−9.7	−9.2	6/2(R)[e]
	0.7	16.0	16.3	−9.0	−9.1	6/2(T)[f]
	2.2	30.8	33.4	−8.3	−8.7	7/5(V)[g]
	1.4	20.4	21.2	6.4	6.2	6/2(V)[h]
	1.0	18.3	18.5	−111.2	−11.4	7/2(V)[i]
Ketoprofen	5.7	12.3	14.3	−4.4	−6.2	6/2(R)[d]
	6.1	19.5	23.3	9.7	8.2	6/2(R)[d]
	1.1	12.5	13.0	−9.3	−9.8	6/2(T)[f]
	4.5	27.7	40.3	−7.3	−9.4	7/5(V)[g]
	4.0	18.0	21.0	7.3	6.3	6/2(V)[h]
	1.7	17.4	17.8	−10.6	−10.6	7/2(V)[i]
Dansyl-amino acids						
						
Phenylalanine	9.9	10.1	13.3	−5.6	−6.9	6/2(R)[d]
	8.6	26.5(D)	38.5(L)	7.2	4.9	6/2(R)[e]
	4.8	20.9(D)	24.8(L)	−4.5	−5.7	7/5(V)[g]
	3.6	35.4	43.2	3.7	3.0	6/2(V)[h]
	1.8	17.9	18.5	−10.5	−10.9	7/2(V)[i]
Valine	2.0	13.8	14.6	−5.8	−6.5	6/2(R)[d]
	7.7	25.2(D)	27.5(L)	7.5	6.9	6/2(R)[e]
	9.6	22.8(D)	33.3(L)	−5.0	−7.3	7/5(V)[g]
	10.6	27.3	37.5	4.8	3.5	6/2(V)[h]
	2.1	16.5	16.9	−10.2	−10.5	7/2(V)[i]
Other carboxylic acid compounds						
Indoleacetic acid	3.4	12.5	17.1	−8.7	−12.2	6/2(R)[d]
	0.5	14.3	14.5	−10.3	−10.5	6/2(T)[f]
	1.7	36.1	38.6	−11.6	−11.8	6/2(V)[g]

Table 2. Continued

Compound	R_s	Time(1)[a]	Time(2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]	pH/m*M*/C.S.
2-Methoxymandelic acid	0.8	21.0	23.0	−13.2	−13.8	6/2(R)[d]
	2.4	15.3	15.8	12.0	12.0	6/2(V)[e]
2,4-Dinitrophenyl-amino acid						
						
Norleucine	15.2	22.6	33.1	−2.1	−4.3	7/2(R)[dj]
	3.4	12.0	13.8	−6.8	−8.5	7/2(T)[f]
	0.8	40.5	41.5	−8.5	−8.6	6/5(V)[g]

[a] Migration time (in min) of first-eluting enantiomer.
[b] Migration time (in min) of second-eluting enantiomer.
[c] $\mu_e(1)$ and $\mu_e(2)$ are the effective electrophoretic mobilities of the first and second eluting enantiomer in $cm^2\ kV^{-1}\ min^{-1}$.
[d] Run voltage +5 kV. See Ref. [41].
[e] Run voltage −10 kV in coated capillary column.
[f] Run voltage +5 kV. See Ref. [36].
[g] Run voltage +5 kV. See Ref. [39].
[h] Run voltage −10 kV in coated capillary column. See Ref. [45].
[i] Run voltage +5 kV with 21 m*M* SDS added to the run buffer. See Ref. [42].
[j] With 30% 2-propanol added to the run buffer.

modification of vancomycin structure can provide significantly different selectivity for some solutes. The macrocyclic antibiotic A82846B, an analogue of vancomycin, was used for the separation of the non-steroidal anti-inflammatories, naproxen and flurbioprofen, and the dansyl amino acids tryptophan and valine [49]. A82846B differs from vancomycin in two ways: (1) its disaccharide amino sugar is epimeric, and (2) it contains an additional epi-vancomsamine (see Fig. 1b). A82846B has an isoelectric point at pH 9, reflecting its basic character, and the additional amine group results in a greater positive fractional charge at pHs commonly employed in CE with vancomycin (i.e., below pH 7). The authors found A82846B to produce superior separations than vancomycin for flurbiprofen, dansylvaline, and dansyltryptophan. As with other chiral selectors, small structural differences can result in significant differences in selectivity for enantiomers.

3.1.2. The ansamycins — rifamycins B and SV

Rifamycin B is negatively charged and can enantiomerically resolve cationic compounds such as protonated amines making it complementary to the glycopeptide antibiotics, which are particularly adept at resolving anionic compounds. A series of pharmacologically active amino alcohols have also been successfully resolved using rifamycin B as the chiral selector [38]. These include adrenergics, vasoconstrictors, bronchiodilators, vasodilators, and β-adrenergic blockers. Enantioselectivity is greatest for compounds in which a hydroxyl group is α to an aromatic ring rather than β or γ and secondary amines exhibit better enantiorecognition than primary amines. While rifamycin B was adept at separating single ring structures and had previously been shown to exhibit no enantioselectivity towards double ring or larger structures Ward and coworkers were able to resolve pindolol and propranolol even though both contain multiple ring structures [44]. While pindolol was poorly resolved, propranolol exhibited good resolution, $R_s = 1.3$. It is of interest to note that the two compounds contain one identically substituted ring and differ only in that pindolol is

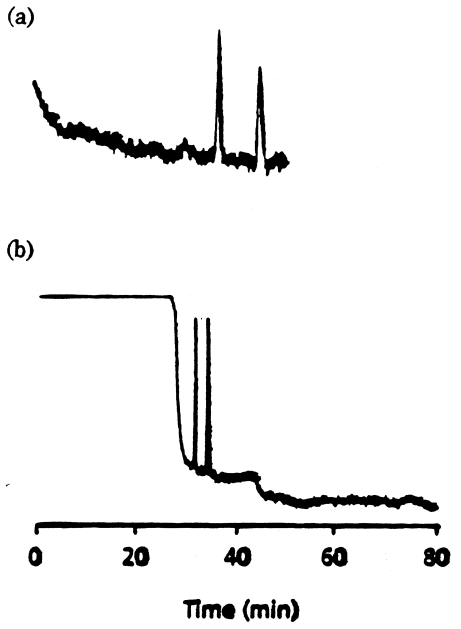


Fig. 4. Electropherograms showing the resolution of racemic dansyl glutamic acid in (a) uncoated capillary and (b) coated capillary. Conditions in uncoated capillary were 2 m*M* vancomycin in 0.1 *M* phosphate buffer at pH 5. The applied potential was +10 kV and detection at 340 nm. Conditions in coated capillary with suppression of electroosmotic flow were 2 m*M* vancomycin in 0.1 *M* phosphate buffer at pH 6. Voltage was −10 kV, direct detection at 254 nm. (Reprinted with permission from Ref. [46], 1966 Advanstar Publication).

composed of an indole instead of a naphthyl ring. This further illustrates the point that structure type and size obviously play an important role in enantioselectivity. It also demonstrates that two ring and possibly larger solutes can be resolved using rifamycin B. The ansamycin rifamycin SV has been shown to be capable of separating negatively-charged solutes is complementary to rifamycin B which is effective for resolving solutes possessing a protonated amine. Rifamycin SV is enantioselective for compounds containing at least two rings such as aspartic acid, hexobarbital and gluethimide [44].

When using the ansamycins with indirect detection, resolution and peak-to-peak separation are significantly affected by the amount of analyte loaded onto the column [44]. By examining separations performed under identical conditions by indirect detection at 275 nm and 350 nm, it has been demonstrated that analyte sensitivity is much greater at 350 nm than at 275 nm. This is attributed to the fact that the baseline noise is substantially lowered at the absorption minima (see Fig. 2), which results in improved sensitivity. Therefore by decreasing the amount of analyte loaded on the capillary column, approximately 80% less in most cases and working at 350 nm (greatest UV minima), enantioresolutions can be substantially improved [44]. Using indirect detection it is possible to measure as little as 0.1% of one enantiomer in the presence of the other using the ansamycins as chiral selectors demonstrating their feasibility for enantiomeric purity assay.

4. Micelle mediated separations using the macrocyclic antibiotics

The use of micelles in conjunction with macrocyclic antibiotics extends the scope of these enantioseparations to neutral solutes and often provides unique selectivity. To date, only the glycopeptide antibiotics have been used in conjunction with micelles. Armstrong and Rundlett have shown that addition of sodium dodecyl sulfate (SDS) micelles with vancomycin to the run buffer enhances efficiency, decreases migration times, and reverses the enantiomeric elution order for some solutes [42]. The addition of SDS micelles to the run buffer can also increase the available elution window for neutral solutes, thereby facilitating the separation of neutral racemates. For vancomycin, in the absence of SDS, the separation of six dansyl-amino acids show that the D-enantiomers elute together before the L-enantiomers, and most peaks are not resolved as shown in Fig. 5 [42]. However, when vancomycin is used above the critical micelle concentration (CMC) (50 m*M*), all of the D-isomers are baseline separated from the L-isomers in the electropherogram. This unique selectivity arises because the relative elution order of the solutes changes from one based on electrophoretic mobility in the vancomycin system to one based on the partition between three pseudophases (i.e., the vancomycin–SDS mixed micelle, the free vancomycin, and the bulk aqueous solution) as shown in Fig. 6. In the micelle system, it is estimated that approximately 90% of vancomycin is bound to the micelle, leaving only 10% free in the solution [42]. As the concentration of SDS increases

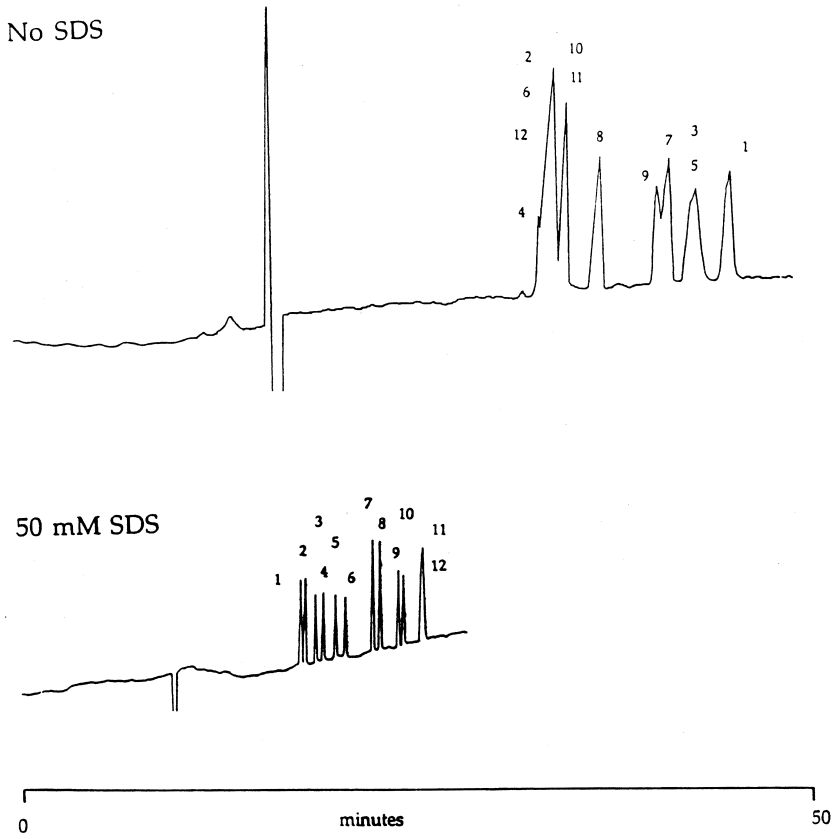


Fig. 5. Electrophorograms showing the effect of SDS on the enantioseparations of dansyl D,L-amino acids in 2 m*M* vancomycin in 50 m*M* phosphate buffer (pH 7) at 20°C. The capillary column used was 50 μm×30 cm, 37 cm in total length. The run voltage was +5 kV and the solutes were detected at 254 nm. The peak designations are: 1. (L)-α-amino-*n*-butyric acid; 2. (D)-α-*n*-butyric acid; 3. (L)-valine; 4. (D)-valine; 5. (L)-valine; 6. (D)-norvaline; 7. (L)-leucine; 8. (D)-leucine; 9. (L)-norleucine; 10. (D)-norleucine; 11. (L)-tryptophan; and 12. (D)-tryptophan. (Reprinted with permission from Ref. [42], 1995 American Chemical Society).

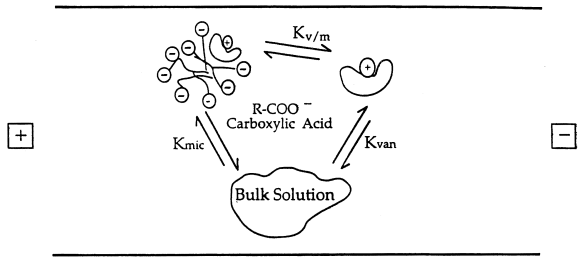


Fig. 6. Representation of the electrophoretic mobility of the analytes, chiral selector and mixed micelles, showing the equilibria of analytes between three pseudophases (bulk aqueous solution, free vancomycin, and mixed micelles). (Reprinted with permission from Ref. [42], 1995 American Chemical Society).

in these systems, the migration time also increases, and the effective mobilities of the analytes become more negative as the solutes partition into the micelles. One potential disadvantage is that enantiomeric resolutions obtained with vancomycin alone are far greater than in an analogous system containing micelles [42]. This loss in resolution is probably caused by the competition of SDS monomer with analyte for the chiral selector. It is noteworthy to mention that the separation occurs via a concurrent process rather than a countercurrent process.

The addition of SDS to a teicoplanin–based separation produces similar effects to vancomycin experiments [36,42]. It should be noted that reversal

of the elution order of enantiomers in teicoplanin systems is not universal. There appears to be a relationship between the size of the analytes binding constant to teicoplanin and the probability of elution reversal. Analytes with larger binding constants to teicoplanin often demonstrate a reversal in elution order while those with smaller binding constants do not. In the case of ristocetin A, the addition of SDS to the analogous conditions as in vancomycin and teicoplanin does not produce the extensive increase in efficiency and decrease in migration times. Furthermore, there appears to be a significant concentration-dependent effect on the selectivity and reversal of elution order is uncommon [43].

5. CE separations in coated capillaries

Using coated capillaries in which electroosmotic flow is suppressed, provides another means to change the selectivity compared to separations performed on uncoated or virgin columns. Besides the ability to alter selectivity there are several features associated with this technique that are advantageous. The strongly absorbing nature of the macrocyclic antibiotics in the UV region of the spectrum can make resolving enantiomers quite difficult at times, even at their effective concentrations. With the glycopeptides at the low enough concentrations, where direct detection is possible, sensitivity can be poor and detection difficult for some compounds that lack conjugated ring system. These disadvantages can be reduced and in some cases eliminated with coated capillary columns. The effectiveness of ristocetin A and vancomycin as chiral selectors in coated capillaries has been demonstrated using a countercurrent process for separations [8,45].

The countercurrent separation process is shown in Fig. 7. After the column is totally filled, the solute is injected immediately. The positively-charged vancomycin migrates towards the cathode (at the injection end), as the solutes migrates towards the anode (the detection end) in a countercurrent process. Separation is achieved and the solutes reach the detection window after the chiral selectors vancomycin or ristocetin A, moving in the opposite direction, have cleared the window. This technique not only improves sensitivity but aids quantification

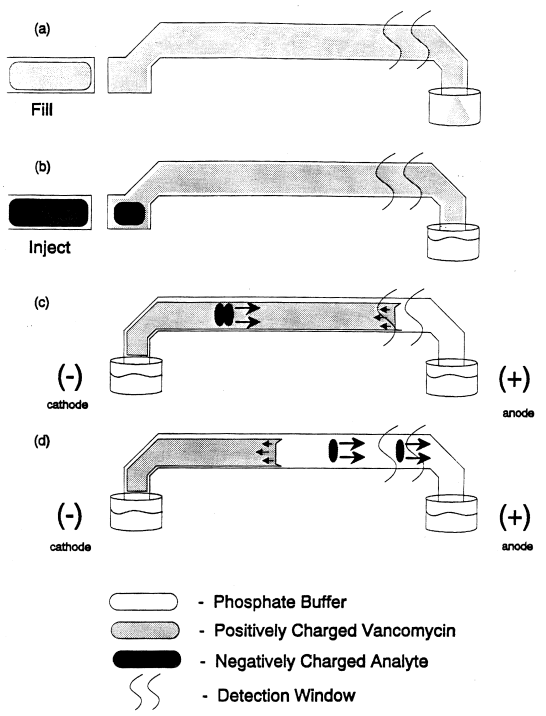


Fig. 7. Schematic showing the counter-current process with suppression of electroosmotic flow: (a) the column is filled with running buffer containing chiral selector; (b) the sample is loaded into the capillary; (c) a potential is applied across the capillary, creating a countercurrent process in which the chiral selector and analyte migrate in opposite directions; and (d) each isomer reaches the detection window after the chiral selector has passed. (Reprinted with permission from Ref. [45], 1996 John Wiley & Sons).

because the elution of solutes occurs after the background absorbance has returned to minimum.

By use of this technique, racemic compounds including the non-steroidal anti-inflammatories and several dansyl-amino acids have been successfully separated [8,45]. Table 2 lists a number of compounds separated using the countercurrent process. Baseline resolution was obtained for all the compounds examined at 2 m*M* vancomycin, 0.1 *M* phosphate buffer at pH 6. The baseline separation of racemic dansylglutamic acid is shown in Fig. 4b. The dansyl-amino acids usually exhibited better enantioselectivity in this system than the non-steroidal anti-inflammatories. This is probably due to the fact that the dansyl groups contain a tertiary amine, which is capable of additional hydrogen bonding, which the non-steroidal anti-inflammatories lack.

Since the chiral selector clears the detection window before the solute is detected, the signal-to-noise ratio is substantially improved, hence increasing the sensitivity of this technique. The use of higher concentrations of macrocyclic antibiotics in the running buffer is not precluded since detection occurs after the selector clears the window. For example, the use of rifamycin B in uncoated columns is limited since the background absorbance becomes substantial, resulting in prohibitively small signal-to-noise ratios; whereas with a countercurrent process, concentrations higher than 30 m*M* could be used in the running buffer. Other advantages of this technique include, in contrast to an uncoated column, the elution order of some enantiomers can be reversed, efficiency is improved due to a reduction of wall adsorption effects and only a minuscule amount of chiral selector is consumed.

As previously stated, because separations in the countercurrent process occur by different mechanisms, it is possible to reverse the elution order of many compounds as opposed to their elution order in an uncoated column. In an uncoated column, the enantiomer that binds most strongly to vancomycin will migrate through the column first. In the countercurrent process with a coated column, the negatively-charged analyte migrates towards the anode (detection end) and the positively charge vancomycin migrates towards the cathode (injection end). In the absence of electroosmotic flow, the enantiomer, which binds most strongly to vancomycin, will have its migration time retarded and will elute last from the capillary column. This retention order is opposite from the retention order observed when an uncoated column is used, in which the analyte that binds most strongly to vancomycin elutes first.

6. Selecting operating parameters in macrocyclic antibiotic based separations

Several parameters such as concentration of the chiral selector, pH, addition of organic modifiers or other additives, and buffers have been found to have an important influence on the resolution. We will first begin by examining the effect of each operating parameter according to its impact on the separation.

6.1. Concentration of chiral selector

The concentration of chiral selector is an easily adjusted parameter that has a profound impact on resolution and migration time. In general, increasing the concentration of the chiral selector, increases enantiomeric resolutions and migration times [8,39,41,43,45]. Intuitively it might be expected that the migration times of solutes should decrease as the chiral selector concentration is increased when using uncoated capillaries. This would be expected since the positively-charged chiral selector migrates towards the cathode (outlet), and the negatively-charged analytes towards the anode (inlet) and as chiral selector concentration is increased complexation between analyte and selector increases. However, such phenomenon has not been observed with any of the glycopeptides studied [39,41,43]. Several factors which are not readily apparent need to be considered to explain the direct proportionality between migration time and chiral selector concentration. For example, as the concentration of vancomycin increases in the run buffer, the electroosmotic flow velocity decreases substantially. Three factors are responsible for this observation. The predominant reason is that vancomycin adsorbs onto the wall of the capillary and suppresses the electroosmotic flow. Secondly, once adsorbed to the capillary wall it acts as a pseudostationary phase by retarding the solute migration. Lastly, increasing the quantity of vancomycin increases the viscosity of the solution, further decreasing electroosmotic flow though this effect is expected to be minimal at the concentrations employed. Thus, as chiral selector concentration is increased, the decrease in electroosmotic flow coupled with the chiral selector acting as a pseudostationary phase retarding the analyte, dominate and have the overall effect of decreasing migration times as chiral selector concentration is increased.

Ristocetin A, teicoplanin, and vancomycin, differ in several of their separation characteristics with respect to chiral selector concentration. At chiral selector concentrations of 2–5 m*M*, ristocetin A-based separations display significantly smaller migration times than analogous separations using vancomycin [41,43]. This is because vancomycin adsorbs to the uncoated capillary wall much more

strongly than ristocetin A, which has bulkier pendant saccharide moieties. For any given analyte under comparable conditions, migration times for vancomycin is usually twice those of ristocetin A; teicoplanin results in intermediate migration times. For example, the enantiomers of mandelic acid eluted in approximately 23.0 min with ristocetin A, 51.5 min with vancomycin, and 31.5 min with teicoplanin [43].

Similar to the glycopeptide antibiotics, the concentration of a chiral selector also has a profound impact on resolution and migration times in the ansamycins. Again, in general, increasing chiral selector concentration results in an increase in enantioresolution and an increase in migration times due to a decrease in electrophoretic mobility [38,44]. Since the charge on the chiral selector is negative and the analyte is positively-charged, the chiral selector migrates towards the inlet, thus the greater the degree of complexation between analyte and chiral selector the greater the increase in migration times. Secondly, at the much higher effective concentrations employed (20–25 m*M*), viscosity effects are much greater than in the case of the glycopeptides. Chiral selector concentrations are limited to about 25 m*M* since increasing the chiral selector concentration much above 25 m*M* results in an unacceptably small signal-to-noise ratio due to the strong UV absorption of the chiral selector.

The chiral selector concentration also has a significant effect on separation when using the countercurrent technique with coated capillaries. With coated capillaries, an increase in vancomycin concentration usually causes an increase in resolution, a slight increase in migration times, and a decrease in effective mobilities for the compounds studied [8,45]. This observation is consistent with a countercurrent process: as the degree of interaction with vancomycin is increased, the mobility of solutes (bound to vancomycin) decreases because vancomycin and the solutes are travelling in opposite directions. Since the capillary wall is coated, electroosmotic flow is suppressed and there is little or no interaction between vancomycin and the capillary wall. Vancomycin appears to affect the migration times and mobilities of the second eluting isomer greater than the first eluting isomer for the non-steroidal anti-inflammatories. This indicates that for the non-steroidal anti-inflammatories, the first eluting isomer binds weakly to vancomycin, and separation largely occurs as a result of binding between vancomycin and the second eluting isomer.

6.2. pH

The effect of pH on enantioseparations can be significant when ionizable chiral selectors such as glycopeptide antibiotics or ansamycins are involved. In such cases, the pH of the running electrolytes govern not only the charge and migration behavior of the analytes, but that of the chiral selectors as well [38,39,41]. In general with the glycopeptides, lower pH values within the range of 4 to 9 produce better enantiomer resolutions in comparison to higher pH values. For most solutes, increasing the pH from 5 to 8 decreases the enantioselectivity, with the most significant decrease occurring between pH 6 and pH 8. It is important to note that at pH lower than 4 or above 9, ristocetin A, teicoplanin, and vancomycin are not stable in solution.

Enhanced enantioselectivities can be achieved when the electromigrations of the chiral selector and chiral solutes are opposite to one another [39,44]. For the ansamycin rifamycin B, the enantioresolution increased with increasing pH, with a maximum at pH 7. A decrease in enantioresolution is observed with increasing pH above pH 7. This phenomenon can be explained in terms of charge of the chiral selector and the analyte being studied. For example, consider a system in which rifamycin B is used as a chiral selector to separate an amine-containing analyte. As pH is lowered, rifamycin B, a dibasic acid, loses some of its negative charge, which precludes a strong charge-to-charge interaction with the positive charge analyte. As pH approaches 7, however, rifamycin B exists as a di-anion while the analyte is still positively charged. Under this condition, a strong electrostatic interaction can take place. At pH higher than 7, again, the amine-containing analyte is deprotonated, leaving the analyte negatively charged, which precludes strong electrostatic interaction. Although other parameters such as electroosmotic flow can affect enantioseparation, in such cases it is clear that electrostatic interaction plays a prominent role in enantioresolution.

6.3. Organic modifiers

Although organic modifiers can enhance resolutions for some analytes, the effects are small in comparison to other factors, i.e., chiral selector concentration and pH. Of the organic modifiers investigated, 2-propanol consistently appears to exhibit the greatest influence on separations for the macrocyclic antibiotics [38,39,41,44]. In the case of rifamycin B and SV, no enantioselectivity can be observed in the absence of organic modifier in the run buffer. The greatest enhancement in enantioresolution occurred with 2-propanol and increasing the 2-propanol concentration increased resolution, decreased electrophoretic mobilities, and thereby increased migration times. While the action of organic modifiers with respect to separation is not clearly understood, in the case of the ansamycins which are surface active, the addition of organic modifier disrupts their aggregation which may enhance their enantiorecognition ability.

The addition of organic modifiers have been shown to enhance enantioselectivity when using the glycopeptides as well but the effects are not as dramatic as those reported with the ansamycins. The enantioselectivity in ristocetin A-based separations is affected by the addition of organic modifier to a larger extent than the other two glycopeptides [41,43]. For example, the resolution of ketoprofen in 2 m*M* ristocetin A, 0.1 *M* phosphate buffer (pH 7) increases from 2.6 to 5.7 when 20% 2-propanol is added to the run buffer. This causes the migration time to increase from 12.6 to 26.3 min as a result of the increased viscosity and decreased electroosmotic flow [41]. Conversely, vancomycin-based enantioseparations are rarely improved and are sometimes degraded upon the addition of small amounts of miscible, organic co-solvents to the run buffer [39]. In the case of teicoplanin, the addition of organic modifier has a more ambiguous effect than in vancomycin and ristocetin A. This may be due to the alteration or destruction of teicoplanin self-aggregates. The enantioresolutions of most compounds can be enhanced by the addition of 10%–30% acetonitrile, but in many cases no conclusive results on the influence of organic modifier can be established. For example, adding 10% acetonitrile to 2 m*M* teicoplanin in 0.1 *M* pH 6.0 phosphate buffer improves enantioresolution of mandelic acid from 2.2 to 3.4, while for trans-4-cotinine carboxylic acid enantioresolution drops from 1.4 to 1.1 upon the addition of 10% acetonitrile [36]. In general with the macrocyclic antibiotics, as the organic modifier concentration is increased, a leveling effect with respect to resolution is observed, and a further increase in organic modifier (>40%) results in decreasing enantiomer resolution.

6.4. Miscellaneous

In addition to other parameters previously discussed, background electrolyte also plays a significant role in enantioseparations. The mobilities of buffer ions can have an effect on enantioresolutions and peak efficiencies. Increasing the concentration of the background electrolyte, i.e., increasing ionic strength of the buffer, coupled with increasing the applied voltage, can cause a decrease in separation time [38,43]. This results from reducing the interactions between the glycopeptides and the capillary wall. One disadvantage is that increasing the background electrolyte generally causes an increase in Joule heating, which can result in a decrease in separation efficiency. The increase in Joule heating can become an important factor when the glycopeptide antibiotics are employed as chiral selectors. Since the glycopeptide antibiotics are less stable at higher temperatures (see Section 2.4), in most CE works involving the glycopeptides as chiral selectors the background electrolyte concentration used tended to be higher than usual in order to compromise between ionic strength and lower voltages. In enantiomeric separations using the ansamycins, the optimum concentration of phosphate buffer appears to be approximately 0.1 *M*. Also, the addition of other buffering components such as borate has been shown to inhibit the separations. More research must be conducted to fully understand the effect of background electrolyte on enantioresolutions.

7. Conclusions

Macrocyclic antibiotics, notably the glycopeptides and the ansamycins, have demonstrated unique and powerful abilities as chiral selectors. Although ris-

tocetin A, teicoplanin, and vancomycin have structural similarities as glycopeptides, they often differ in their enantiorecognition properties. While the glycopeptide antibiotics demonstrate similar, but not identical, selectivity towards enantiomers, the ansamycins rifamycins B and SV are complementary to each other. By use of either micelle-mediated (e.g., SDS) separations or a countercurrent process with suppression of electroosmotic flow, the elution order of enantiomers may be reversed. The countercurrent technique offers several advantages over conventional CE mode, i.e., improved sensitivity, efficiency, and minuscule consumption of chiral selector. Enantioselectivities of the macrocyclic antibiotics in CE also can be altered by changing various separation parameters. Generally, an increase in chiral selector concentration results in an increase in migration times and selectivities. For most solutes, an increase in pH range from 5 to 8 causes a decrease in enantioselectivity, with the most significant reduction between pH 6 and pH 8. Addition of organic modifier appears to impact enantioseparations of the ansamycins more than the glycopeptide antibiotics. Of the organic modifiers investigated, 2-propanol appear to exhibit the greatest influence on both glycopeptide- and ansamycin-based separations. Though the effect of organic modifier affects enantioselectivity to a lesser extent than other parameters such as chiral selector concentration and pH. Of the three glycopeptide antibiotics (ristocetin A, teicoplanin, and vancomycin), ristocetin A appears to resolve the greatest variety and number of racemates. Without a doubt much work still needs to be done to fully explore the effects of various parameters on separation and to understand the underlying separation mechanisms for both current and newly developed techniques. The development of more innovative chiral selectors will enhance the scope of enantioseparations and will ensure the continued growth of chiral separations using CE.

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