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Highly enantioselective capillary electrophoretic separations with dilute solutions of the macrocyclic antibiotic ristocetin A

Daniel W. Armstrong*, Mary P. Gasper, Kimber L. Rundlett

Department of Chemistry, University of Missouri–Rolla, Rolla, MO 65401, USA

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Abstract

Ristocetin A is one of a series of structurally related amphoteric, glycopeptide, macrocyclic antibiotics. These compounds have several features that make them attractive as chiral selectors. These include spatially oriented functional groups that are known to provide the types of interactions that are conducive to enantio-recognition, a somewhat rigid "pocket" that can provide a site for hydrophobic interactions and polar, flexible arms (*i.e.*, pendent sugar moieties) that can rotate to hydrogen bond and otherwise interact with a variety of chiral analytes. In addition, these compounds are sufficiently soluble in water, aqueous buffers and aqueous–organic solvents that are commonly used in capillary electrophoresis (CE). The use and optimization of ristocetin A as a chiral selector in CE is discussed. Over 120 racemates are resolved including a variety of N-blocked amino acids, non-steroidal anti-inflammatory compounds and a large number of biologically important compounds containing carboxylic acid groups (e.g., mandelic acid derivatives, lactic acid derivatives, folinic acid, tropic acid).

1. Introduction

Recently, several macrocyclic antibiotics and their derivatives have been shown to be exceptional chiral selectors in capillary electrophoresis (CE) [1–3], HPLC [3,4] and TLC [5]. Because of the large number of these compounds and their structural variations, it is expected that they will have a significant impact on many types of enantiomeric separations. For example, ansamycins such as rifamycin B were used to resolve effectively a number of racemic amino alcohols using CE [1]. Conversely, vancomycin, which contains three small macrocyclic rings, effectively resolved over 100 negatively charged analytes, most of which contained at least one carboxylic acid functional group [2]. Vancomycin was also an effective chiral mobile phase additive for the reversed-phase TLC separation of enantiomers [5]. When bonded to 5-μm silica gel and packed in columns, rifamycin B, vancomycin and thiostrepton proved to be effective chiral stationary phases [3,4].

It appears that macrocyclic antibiotics may be more widely effective in CE than most other currently used chiral selectors. A review describing the development of chiral media for CE and future trends appeared recently [6]. Another new CE chiral selector, heparin, was also shown to resolve effectively several cationic compounds [7]. In this paper we describe a different mac-

* Corresponding author.

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rocyclic chiral selector, ristocetin A, and demonstrate that it is very useful in the CE resolution of a variety of racemic analytes.

Ristocetin A is produced as a fermentation product of *Nocardia lurida* [8–10]. As an antibiotic it is very active against Gram-positive bacteria including strains that are resistant to other antibiotics [9,10]. Its action results from selective binding to terminal D-Ala–D-Ala sequences in mucopeptides and inhibiting bacterial cell wall synthesis [11,12]. *Norcardia lurida* also produces a related component referred to as ristocetin B, which differs in the number of sugars in the side-chains. Ristocetin A has a molecular mass of 2066 and consists of an aglycone portion with four joined macrocyclic rings (two having sixteen members, one with fourteen members and one with twelve members) to which several sugars (e.g., arabinose, glucose, mannose and rhamnose) are covalently attached. The structure of ristocetin A is shown in Fig. 1. The ristocetins are related to other known macrocyclic antibiotics including vancomycin, β-avoparcin and teicoplanin [2,12]. As can be seen, ristocetin A has the functionalities and geometry that tend to accentuate chiral recognition between molecules in solution. This includes 38 stereogenic centers, seven aromatic rings, six amide bonds, 21 hydroxyl groups, two primary amine groups and one methyl ester. Together the four macrocyclic rings form a "basket"-like structure. This amphoteric compound is soluble in acidic aqueous solutions and less soluble at neutral pH. It is fairly soluble in polar aprotic solvents such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF), but insoluble in non-polar organic solvents.

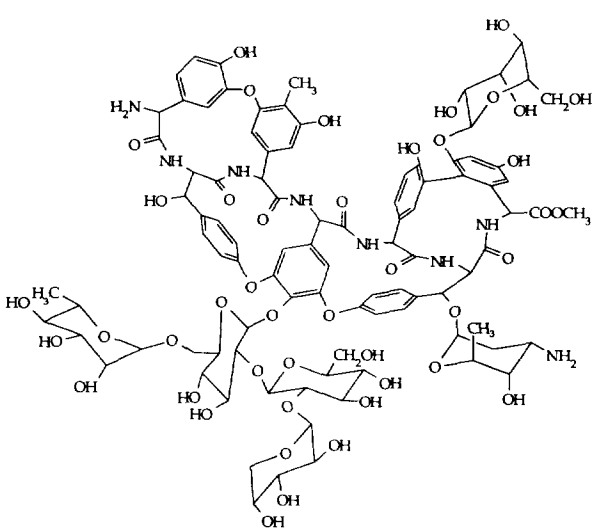


Fig. 1. Structure of ristocetin A ($M_r = 2066$) showing the "pocket" formed by the four fused macrocyclic rings and the polar, pendent carbohydrate moieties.

In this work, we evaluated the chiral recognition properties of ristocetin A using CE. Its suitability as a chiral selector for enantiomeric separations and its similarities to and differences from other related chiral selectors are discussed.

2. Experimental

2.1. Materials

All commercially prepared amino acids, amino acid derivatives, non-steroidal anti-inflammatory compounds, sodium dihydrogenphophate, potassium hydroxide, sodium hydroxide, amethopterin, folinic acid and ristocetin sulfate salt were purchased from Sigma (St. Louis, MO, USA). *p*-Chloromandelic acid was obtained from Chem Service (West Chester, PA, USA). All other chiral carboxylic acid compounds were purchased from Aldrich (Milwaukee, WI, USA). 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatizing reagent was supplied by Waters (Milford, MA, USA). 2-Propanol was of HPLC grade from Fisher Scientific (St. Louis, MO, USA).

2.2. Methods

Waters provided the Quanta 4000 capillary electrophoresis apparatus equipped with a fixed-wavelength UV lamp. All chiral separations were performed using a 32.5 cm (25 cm to the detector × 50 μm I.D.) fused-silica capillary obtained from Quadrex (New Haven, CT, USA) and detected at 254 nm. The capillary was prepared daily by conditioning with 0.1 *M* potassium hydroxide solution for 10 min. Next, the

capillary was purged with distilled water for 5 min followed by the appropriate concentration and pH of the running buffer for an additional 5 min. The 0.1 *M* sodium phosphate buffer solutions were prepared in a volumetric flask and adjusted to the desired pH with sodium hydroxide. Ristocetin solution was prepared in a volumetric flask, dissolved in the appropriate phosphate buffer and degassed by sonication. The aqueous buffer–organic modifier (2-propanol) mixture was prepared by volume. All samples were dissolved in distilled water and filtered with a 0.45-μm nylon syringe filter purchased from Alltech (Deerfield, IL, USA) prior to injection. The run voltage for all separations was +5 kV. Samples were hydrostatically injected for 3 or 5 s. Chiral separations were achieved with solutions of 0.1 mg/ml and at ambient temperature (22°C). The derivatization procedure for AQC amino acid compounds has been described previously [13]. The absorbance spectra were measured using a Hitachi Model U-2000 double-beam UV–Vis spectrophotometer.

3. Results and discussion

Ristocetin A has some structural similarities to vancomycin, which was shown previously to be an effective chiral selector in CE, HPLC and TLC [2,4,5]. The aglycone of ristocetin A consists of four fused macrocyclic rings rather than three [2,12]. In addition, ristocetin A has more pendant sugar moieties than vancomycin (Fig. 1). Hence the overall molecular mass of ristocetin A is about 36% more than that of vancomycin. However, vancomycin has two chloro-substituted phenyl rings in the central part of the aglycone that are not present in ristocetin A. As shown in Fig. 2, ristocetin A has zero electrophoretic mobility at pH $\approx$ 7.5 in 0.1 *M* phosphate buffer. This is only slightly higher than that found for vancomycin [2]. Hence at pH $<$ 7.5 ristocetin A is positively charged and migrates in the same direction as the electroosmotic flow. Also, it would tend to interact electrostatically with anionic species.

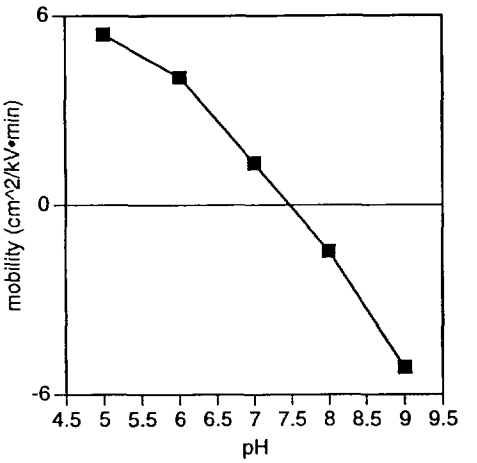


Fig. 2. Plot showing the electrophoretic mobility of ristocetin A at different pH. All runs were performed in 0.1 *M* phosphate buffer (see Experimental) with a 32.5 cm (25 cm to the detector) ×50 μm i.d. fused-silica capillary at a run voltage of +5 kV.

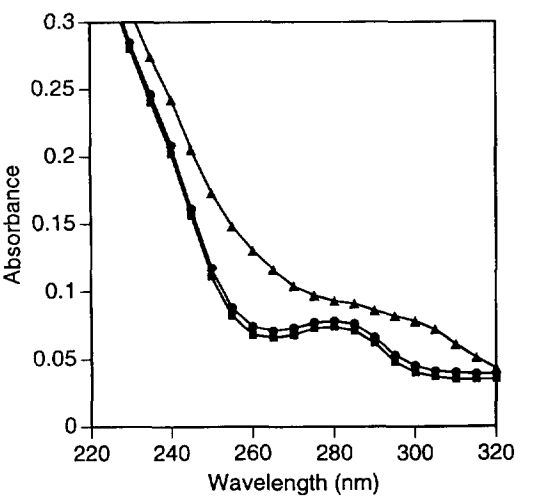


Fig. 3. UV absorbance spectra of ristocetin A (at a concentration of 7.2 $\cdot 10^{-3}$ mg/ml in 0.1 *M* phosphate buffer) at pH 4.0 (■), pH 7.0 (●) and pH 9.9 (▲).

Fig. 3 shows UV spectra of ristocetin A at pH between 4.0 and 9.9. Ristocetin A does not absorb light in the visible region to any significant extent. It is a weak absorber between 250 to 380 nm with a small maximum at ca. 282 nm (Fig. 3). Below 250 nm its absorbance increases significantly. Direct UV detection of aromatic

analytes can be used at wavelengths ≥254 nm. This is because of a combination of two factors: (a) the relatively low absorbance of the chiral selector at these wavelengths and (b) the very low concentrations of the chiral selector needed for the separations (i.e., between 1 and 5 m*M*). At alkaline pH there appears to be a slight red shift of the entire spectrum and a slight levelling of the spectral peaks and troughs (Fig. 3).

Table 1 lists the separation data for over 120 racemic compounds that were resolved using dilute solutions of ristocetin A. This includes all types of N-blocked amino acids (Fig. 4) and a variety of other acidic or anionic compounds. Non-steroidal anti-inflammatory compounds are particularly easy to resolve. The enantioselectivity of ristocetin A appears to be similar to that of vancomycin [2]. However, several chiral compounds containing carboxylic acid functional groups were resolved with ristocetin A that could not be separated with vancomycin. These include mandelic acid, 2-methoxymandelic acid, hexahydromandelic acid, 3-hydroxy-4-methoxymandelic acid, *o*-acetylmandelic acid, 3-methoxymandelic acid, *β*-phenyllactic acid, tropic acid, 2-bromo-3-methylbutyric acid, 1-benzocyclobutenecarboxylic acid and *p*-chloromandelic acid (Fig. 5). Ristocetin A appears to be complementary to rifamycin B and heparin (which resolve cationic compounds) when used as a CE chiral selector [1,7].

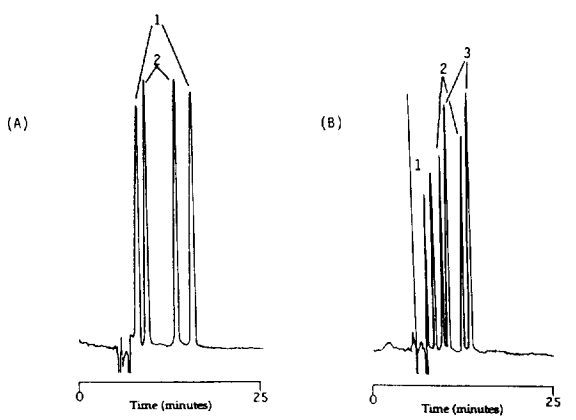


Fig. 4. (A) Electropherogram showing the resolution of racemic (1) N-benzoylalanine and (2) N-benzoylmethionine (B) electropherogram showing the resolution of racemic (1) AQC methionine, (2) AQC *α*-aminopimellic acid and (3) AQC serine. The running buffer was 0.1 *M* phosphate buffer (pH 6)–2 m*M* ristocetin A. The run voltage was 5 kV. See Experimental for further details.

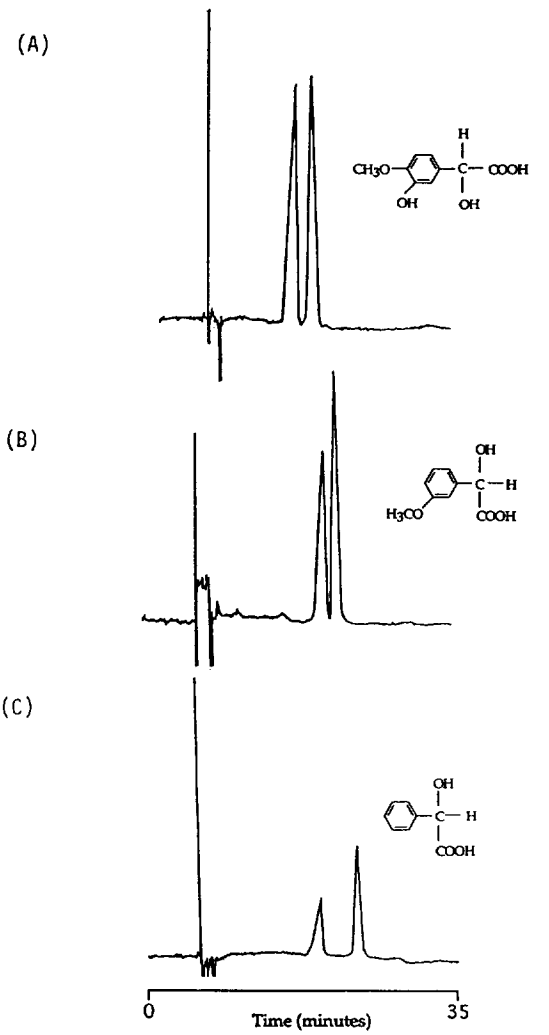
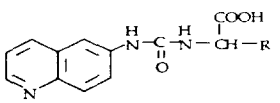
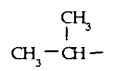
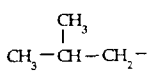
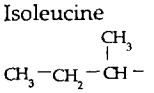
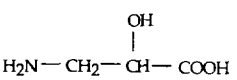
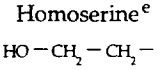
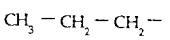
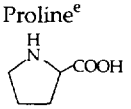
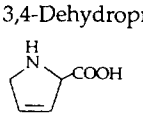
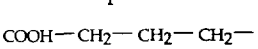


Fig. 5. Electropherograms showing the resolution of racemic (A) 3-hydroxy-4-methoxymandelic acid, (B) 3-methoxymandelic acid and (C) mandelic acid. The running buffer was of 0.1 *M* phosphate buffer (pH 6)–2 m*M* ristocetin A. The run voltage was 5 kV. See Experimental for further details.

There are at least four other distinguishing features of ristocetin A-based CE separations compared with previous vancomycin-based separations. First, at all concentrations between 2 and 5 m*M* ristocetin A (without added organic co-solvent modifiers) the separation times were significantly less than those obtained using van-

Table 1
Enantiomeric resolutions, migration times and apparent and effective mobilities of enantiomers of amino acid derivatives, non-steroidal anti-inflamatory compounds and carboxylic acids separated with ristocetin

Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
AQC Amino acids[d]						
						
Alanine CH_3-	7.0	9.4	8.8 (D)	12.6 (L)	−2.5	−8.0
Valine 	7.0	6.0	10.5	12.6	−5.4	−8.0
Leucine 	7.0	5.4	9.5 (D)	11.3 (L)	−3.9	−6.5
Isoleucine 	7.0	3.8	9.0	10.6	−2.8	−5.6
Isoserine 	6.0	1.2	13.1	13.7	−8.6	−9.1
Homoserine[e] 	6.0	11.1	12.3	16.7	−0.9	−4.4
Norvaline[f] 	7.0	11.3	13.6	19.0	−1.9	−5.3
α-Amino-n-butyric acid 	7.0	11.3	8.7 (D)	13.2 (L)	−2.2	−8.6
Proline[e] 	7.0	2.5	18.1	19.4	−4.9	−5.6
3,4-Dehydroproline 	6.0	4.8	11.0	13.1	−6.2	−8.5
α-Aminopimelic acid 	7.0	4.6	13.0	17.1	−8.4	−11.5

(Continued on p. 290)

Table 1 *(continued)*

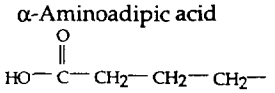
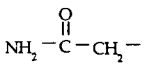
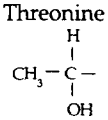
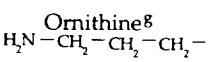
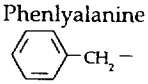
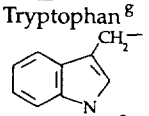
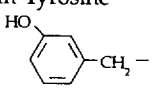
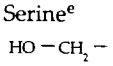
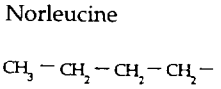
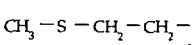
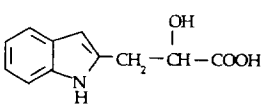
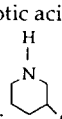
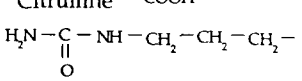
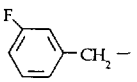
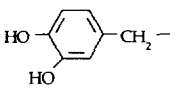
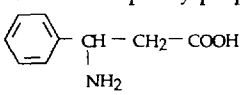
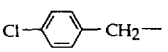
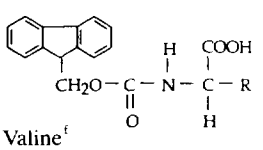
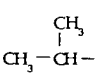
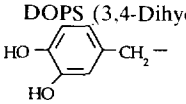
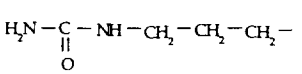
Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)^c$	$\mu_e(2)^c$
α-Aminoadipic acid 	6.0	9.8	13.0	18.1	−5.2	−8.7
Asparagine 	6.0	5.8	11.1	13.6	−6.3	−9.0
Threonine 	6.0	4.2	12.1	13.9	−4.6	−6.4
Ornithine[g] 	7.0	5.3	17.0	19.1	−4.3	−5.3
Phenlyalanine 	7.0	8.4	8.6	10.1	−2.1	−6.1
Tryptophan[g] 	7.0	12.3	17.0	23.2	−4.3	−6.8
m-Tyrosine[e] 	7.0	12.6	12.1	16.2	−0.3	−3.8
Serine[e] 	6.0	10.8	14.6 (D)	19.7 (L)	−2.1	−5.0
Norleucine 	7.0	4.9	9.4	11.3	−1.8	−4.7
Methionine 	7.0	4.0	9.5	11.1	−3.8	−6.3
Indolelactic acid[d] 	7.0	3.5	30.8	35.3	−4.0	−4.7
Nipecotic acid[h] 	7.0	2.0	30.9	32.2	−4.0	−4.2
Citrulline 	7.0	7.2	8.7	11.8	−2.3	−7.1

Table 1 *(continued)*

Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
3-Fluorophenylglycine[f] 	7.0	11.2	12.8	18.0	−1.1	−4.8
3-Fluorophenylalanine[f] 	7.0	10.7	12.8	17.9	−1.1	−4.8
DOPA (Dihydroxyphenylalanine) 	6.0	1.2	12.1	12.5	−7.5	−7.9
3-Amino-3-phenylpropionic acid[f] 	7.0	5.1	11.7	17.4	−2.7	−4.5
4-Chlorophenylalanine[h] 	7.0	15.7	20.2	28.0	−1.2	−3.5
FMOC Amino acids[d]						
 Valine[f]	7.0	9.0	12.1	16.6	−3.6	−7.3
 Alanine[f]	7.0	0.4	14.7	15.0	−6.0	−6.2
DOPS (3,4-Dihydroxyphenylserine)[f] 	7.0	13.8	11.4	18.6	−2.8	−8.4
Citrulline 	7.0	2.6	9.1	10.0	−1.3	−2.8

(Continued on p. 292)

Table 1 *(continued)*

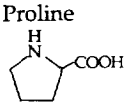
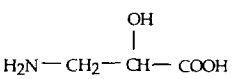
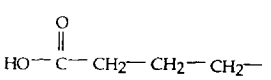
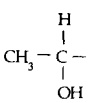
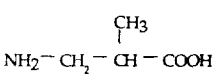
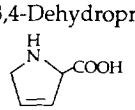
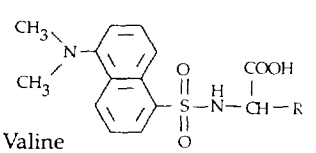
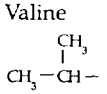
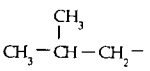
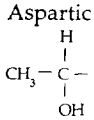
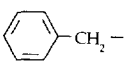
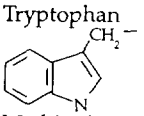
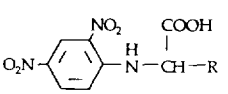
Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
Norleucine $CH_3-CH_2-CH_2-CH_2-$	7.0	0.7	7.4	7.6	−3.1	−3.7
Serine $HO-CH_2-$	7.0	2.4	10.9	12.1	−4.2	−5.6
Proline 	7.0	7.1	9.4	9.9	−1.7	−2.7
Homoserine[f] $HO-CH_2-CH_2-$	7.0	4.4	11.9	13.9	−3.5	−5.5
Isoserine[f] 	7.0	0.7	15.8	16.0	−6.8	−7.0
α-Amino-adipic acid 	7.0	3.4	14.5	16.1	−5.9	−7.0
α-Amino-pimelic acid $COOH-CH_2-CH_2-CH_2-CH_2-CH_2-$	7.0	4.9	16.2	18.7	−7.1	−8.4
Threonine[f] 	7.0	0.9	16.2	16.6	−7.0	−7.3
β-Amino-isobutyric acid[f] 	7.0	0.9	16.6	17.0	−7.3	−7.6
3,4-Dehydroproline 	7.0	1.1	15.1	15.6	−8.4	−8.7
Dansyl Amino acids[d]  Valine 	6.0	2.0	13.8	14.6	−5.8	−6.5

Table 1 *(continued)*

Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
Leucine 	6.0	3.9	11.3	15.6	−3.3	−7.2
Aspartic acid 	6.0	5.6	37.2 (D)	46.5 (L)	−13.3	−14.1
Phenylalanine 	6.0	9.9	10.1	13.3	−1.6	−5.4
Tryptophan 	6.0	7.1	11.6	14.3	−3.6	−6.2
Methionine $CH_3-S-CH_2-CH_2-$	6.0	5.3	12.5 (D)	14.6 (L)	−4.7	−6.5
Norleucine $CH_3-CH_2-CH_2-CH_2-$	6.0	5.3	13.0	15.7	−5.1	−7.3
Norvaline $CH_3-CH_2-CH_2-$	6.0	4.7	12.9	14.6	−5.0	−6.5
α-Amino-n-butyric acid CH_3-CH_2-	6.0	3.9	12.6	14.1	−4.7	−6.1
α-Amino-n-caprylic acid $CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-$	6.0	7.6	10.9	12.9	−2.7	−5.1
Serine $HO-CH_2-$	6.0	1.3	21.1	21.7	−6.2	−6.4

2,4-Dinitrophenyl Amino acids[d]



Methionine $CH_3-S-CH_2-CH_2-$ | 7.0 | 7.1 | 8.2 | 11.0 | −1.0 | −6.2

(Continued on p. 294)

Table 1 *(continued)*

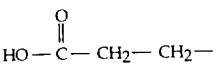
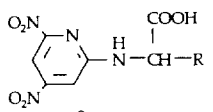
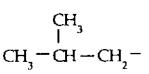
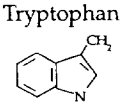
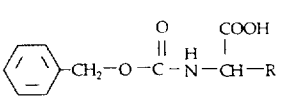
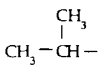
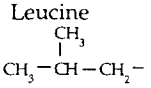
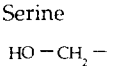
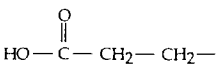
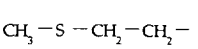
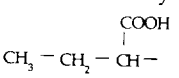
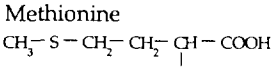
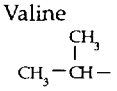
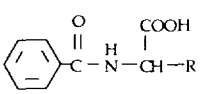
Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
Norleucine[h]						
$CH_3-CH_2-CH_2-CH_2-$	7.0	15.2	22.6	33.1	−2.1	−4.3
Norvaline[h]						
$CH_3-CH_2-CH_2-$	7.0	12.4	20.6	37.6	−1.4	−5.0
α-Amino-n-butyric acid[f]						
CH_3-CH_2-	7.0	3.1	19.0	20.0	−5.1	−5.7
Amino-n-caprylic acid[h]						
$CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-$	7.0	14.7	22.5	29.1	−2.1	−3.7
Glutamic acid						
	7.0	10.8	12.3	22.8	−4.4	−10.5
Ethionine[f]						
$CH_3-CH_2-S-CH_2-CH_2-$	7.0	16.8	22.0	32.5	−2.7	−5.1
N-3,5-Dinitropyridyl Amino acids[d]						
						
Alanine[e]						
CH_3-	6.0	0.4	18.4	20.2	−5.3	−6.1
Leucine						
	6.0	9.0	9.2	13.4	−3.2	−8.8
Serine						
$HO-CH_2-$	7.0	3.1	14.0	15.4	−9.4	−10.4
Tryptophan						
	7.0	9.9	8.9	13.6	−2.7	−5.8
Methionine						
$CH_3-S-CH_2-CH_2-$	7.0	0.8	13.2	13.6	−8.7	−9.0
Carbobenzyloxy Amino acids[d]						
	6.0	6.2	12.6	17.1	−4.7	−8.1
Alanine						
CH_3-						

Table 1 *(continued)*

Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
Valine 	6.0	7.9	10.9 (D)	16.0 (L)	−2.7	−7.5
Leucine 	6.0	3.9	10.4	12.0	−2.0	−4.0
Serine 	6.0	4.8	15.7	18.0	−7.3	−8.6
Glutamic acid 	6.0	2.7	13.6	15.7	−5.7	−7.3
Methionine 	6.0	8.6	11.1 (D)	15.2 (L)	−3.0	−6.9
PHTH Amino acids[d]						
α-Amino-n-butyric acid 	6.0	0.1	14.1	14.5	−6.1	−6.4
Methionine 	6.0	2.7	11.7	13.0	−3.8	−5.1
Valine 	6.0	0.8	15.0	15.4	−6.8	−7.1
N-Benzoyl Amino acids[d]						
Alanine 	6.0	6.1	11.0	20.9	−2.9	−9.9

(Continued on p. 296)

Table 1 *(continued)*

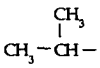
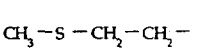
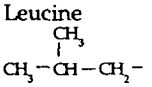
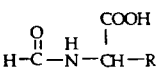
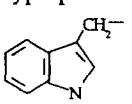
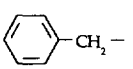
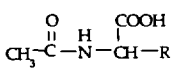
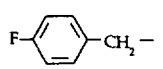
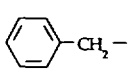
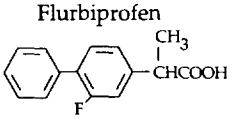
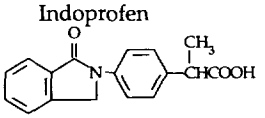
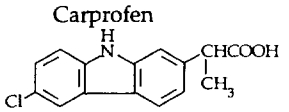
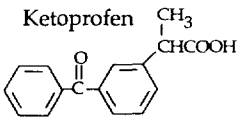
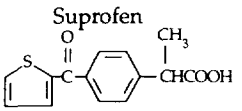
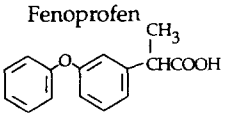
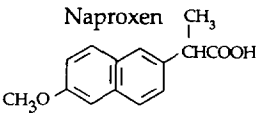
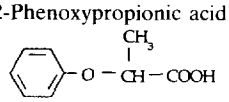
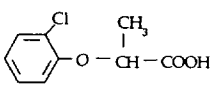
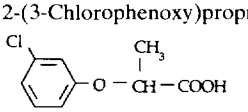
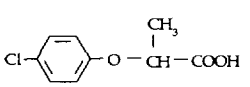
Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
Valine 	6.0	5.5	10.9	20.6	−2.7	−9.8
Methionine 	6.0	6.8	12.1	18.1	−1.3	−5.8
Leucine 	6.0	3.7	11.0	13.6	−6.3	−9.0
N-Formyl Amino acids[d]						
						
Tryptophan 	6.0	5.2	8.1	10.9	−4.9	−6.2
Phenylalanine 	6.0	0.7	14.0	14.6	−3.9	−4.3
N-Acetyl Amino acids[d]						
						
4-Fluorophenylalanine 	7.0	6.7	8.2	13.5	−1.0	−9.0
Phenylalanine 	6.0	0.6	14.2	15.0	−4.0	−4.6
Non-steriodal anti-inflammatory drugs[d]						
Flurbiprofen 	6.0	1.7	27.2	28.1	−8.8	−9.0
Indoprofen 	6.0	1.3	12.7	13.1	−4.8	−5.2

Table 1 *(continued)*

Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
Carprofen 	6.0	1.2	12.1	12.3	−7.4	−7.8
Ketoprofen 	6.0	5.7	12.3	14.3	−4.4	−6.2
Suprofen 	6.0	1.4	17.0	17.4	−8.1	−8.7
Fenoprofen 	6.0	0.9	16.1	16.5	−7.6	−7.8
Naproxen 	6.0	5.8	11.1	12.6	−3.0	−4.7
Other carboxylic acid compounds						
2-Phenoxypropionic acid[d] 	6.0	0.8	21.0	21.7	−13.2	−13.5
2-(2-Chlorophenoxy)propionic acid[d] 	6.0	2.1	19.5	21.4	−12.6	−13.4
2-(3-Chlorophenoxy)propionic acid[d] 	6.0	8.9	11.2	21.4	−3.1	−10.0
2-(4-Chlorophenoxy)propionic acid[d] 	6.0	1.8	15.5	16.9	−10.6	−11.5

(Continued on p. 298)

Table 1 *(continued)*

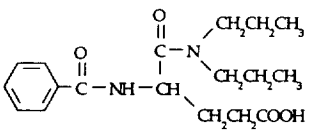
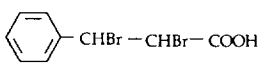
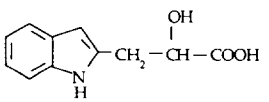
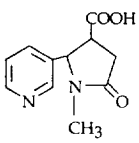
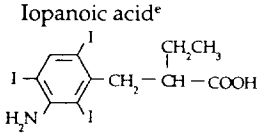
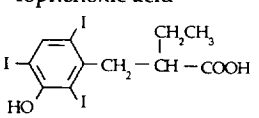
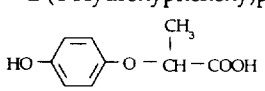
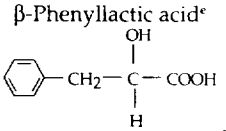
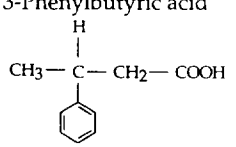
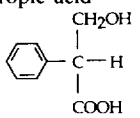
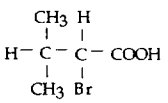
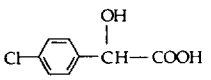
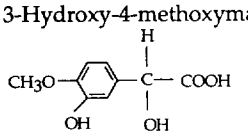
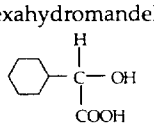
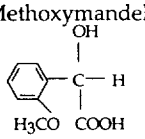
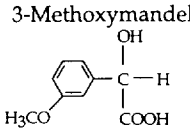
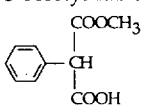
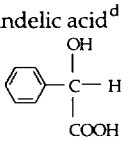
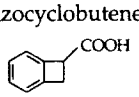
Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
Proglumide[d] 	6.0	1.5	14.1	14.5	−9.4	−9.8
±-Dibromohydrocinnamic acid[e] 	6.0	5.0	20.5	23.4	−6.9	−7.8
Indolelactic acid[d] 	6.0	3.4	12.5	17.1	−8.0	−11.5
±-trans-4-Cotinine carboxylic acid[e] 	6.0	1.6	20.1	21.6	−6.7	−7.3
Iopanoic acid[e] 	6.0	12.3	8.6	11.1	−2.3	−6.5
Iophenoxic acid[e] 	6.0	12.5	10.9	13.4	−6.2	−9.1
2-(4-Hydroxyphenoxy)propionic acid[f] 	7.0	1.0	27.0	29.1	−9.1	−9.2
β-Phenyllactic acid[e] 	6.0	0.7	16.2	16.8	−4.7	−5.1
3-Phenylbutyric acid[d] 	6.0	2.6	15.5	18.7	−10.7	−12.4

Table 1 *(continued)*

Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)^c$	$\mu_e(2)^c$
Tropic acid[d] 	6.0	0.2	21.4	21.8	−13.4	−13.5
2-Bromo-3-methylbutyric acid[d] 	6.0	1.5	25.5	29.0	−14.8	−15.5
p-Chloromandelic acid[g] 	7.0	1.4	17.14	18.4	−11.5	−12.1
3-Hydroxy-4-methoxymandelic acid[d] 	6.0	1.2	24.3	25.8	−11.0	−11.3
Hexahydromandelic acid[e] 	6.0	3.5	23.7	30.1	−10.8	−12.3
2-Methoxymandelic acid[d] 	6.0	0.8	21.0	23.0	−13.2	−13.8
3-Methoxymandelic acid[d] 	6.0	1.5	16.8	20.2	−11.5	−13.0
RS-O-Acetylmandelic acid[d] 	6.0	0.30	22.2	22.5	−13.6	−13.7
Mandelic acid[d] 	6.0	2.0	23.0	28.4	−13.9	−15.2
Benzocyclobutenecarboxylic acid[d] 	6.0	1.1	21.0	22.5	−13.2	−13.7

(Continued on p. 300)

Table 1 *(continued)*

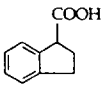
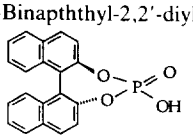
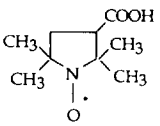
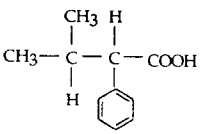
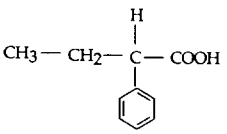
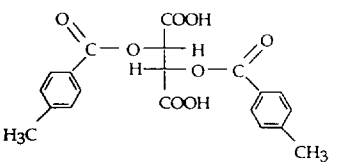
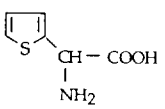
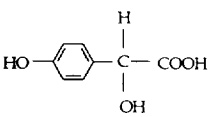
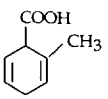
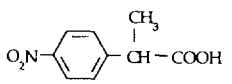
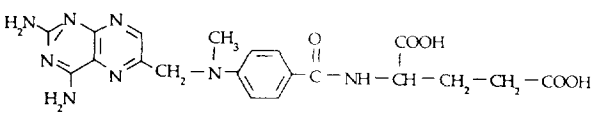
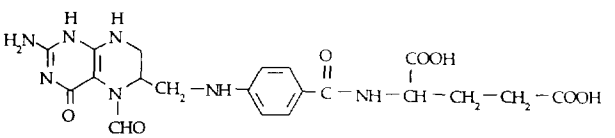
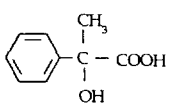
Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
3-Oxo-2-indancarboxylic acid[d] 	6.0	2.6	27.3	32.5	−10.3	−11.3
1,1'-Binapththyl-2,2'-diyl hydrogen phosphate[d] 	6.0	4.11	17.4	19.5	−5.4	−5.6
3-[4-Carbonyl]-PROXYL[d] 	6.0	9.1	26.1	34.3	−12.9	−14.4
3-Methyl-2-phenylbutyric acid[d] 	6.0	6.4	25.0	30.6	−8.3	−9.5
2-Phenylbutyric acid[f] 	7.0	1.24	30.8	32.2	−9.5	−9.7
Di-0,0'-1-Toluoyl tartaric acid[d] 	6.0	8.1	25.8	34.3	−8.5	−10.0
α-Aminothiophenacetic acid[d] 	6.0	9.1	39.2	57.8	−13.9	−15.2
Hydroxymandelic acid[f] 	7.0	5.2	24.0	29.2	−11.3	−12.5

Table 1 *(continued)*

Compound	pH	Resolution	Time (1)[a]	Time (2)[b]	$\mu_e(1)$[c]	$\mu_e(2)$[c]
1,4-Dihydro-2-methylbenzoic acid[d] 	6.0	0.51	36.7	37.6	−10.3	−10.5
2-(4-Nitrophenyl)propionic acid[d] 	6.0	1.6	16.0	16.7	−4.6	−5.1
Amephoterin[g] 	6.0	2.1	32.9	34.6	−6.7	−6.9
Folinic Acid[d,i] 	6.0	1.3	14.8	16.8	−10.0	−11.3
Atrolactic acid[d] 	6.0	0.4	28.6	29.1	−15.3	−15.4

The run voltage for all separations was +5 kV. The pH and concentration of vancomycin are indicated. See the Experimental section for further details.

[a] Migration time (in minutes) of the first-eluting enantiomer.

[b] Migration time (in minutes) of the 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] 2m*M* ristocetin added to run buffer.

[e] 5m*M* ristocetin added to run buffer.

[f] 2m*M* ristocetin added to run buffer with 10% 2-propanol.

[g] 2m*M* ristocetin added to run buffer with 20% 2-propanol.

[h] 2m*M* ristocetin added to run buffer with 30% 2-propanol

[i] This is a diastereomeric separation of folinic acid.

comycin. Separation times with vancomycin as a chiral selector and using analogous conditions were typically between 40 and 70 min [2]. Second, as will be shown, organic modifiers can be used to enhance enantioresolutions substantially when using ristocetin A, but less so with vancomycin [2]. Third, aqueous solutions of ristocetin A appear to decompose more slowly than those of vancomycin [2]. Finally, the current cost of vancomycin is much less than that of ristocetin A.

As shown in Table 2, the concentration of ristocetin A in the running buffer can have a significant effect on the CE separation. In general, higher concentrations of the chiral selector produce higher enantioresolutions and longer

Table 2
Effect of ristocetin concentration on the migration times, effective mobilities and resolution of enantiomers

Ristocetin concentration (mM)	Compound	R_s	$t(1)^a$	$t(2)^a$	$t(\mathrm{EOF})^b$	$\mu_e(1)^c$	$\mu_e(2)^c$
2	2-(3-Chlorophenoxy)-propionic acid	8.9	11.2	21.4	7.6	−3.1	−10.0
	Ketoprofen	5.7	12.3	14.3	7.5	−4.4	−6.2
	3-Methoxymandelic acid	1.5	16.8	20.2	7.6	−11.5	−13.0
	1-Benzocyclobutene-carboxylic acid	1.1	21.0	22.4	7.5	−13.2	−13.7
5	2-(3-Chlorophenoxy)-propionic acid	22.1	11.6	29.3	11.5	−0.2	−8.7
	Ketoprofen	7.4	15.7	27.5	11.5	−3.8	−5.5
	3-Methoxymandelic acid	4.7	19.2	29.9	11.5	−5.8	−8.8
	1-Benzocyclobutene-carboxylic acid	1.8	42.5	49.6	11.5	−10.3	−10.9

The running buffer was 0.1 M phosphate buffer (pH 6) containing the indicated concentration of ristocetin.
[a] The migration times of the enantiomers, $t(1)$ and $t(2)$ are given minutes.
[b] The time corresponding to the electroosmotic flow, $t(\mathrm{EOF})$, is given in minutes. Note that a decrease in the EOF velocity results in an increase in $t(\mathrm{EOF})$.
[c] The effective mobilities, $\mu_e(1)$ and $\mu_e(2)$, are given in $\mathrm{cm^2\ kV^{-1}\ min^{-1}}$.

separation times. The reason for the increase in analyte elution time is that higher concentrations of ristocetin tend to slow the electroosmotic flow (Table 2). This is because at these pHs the positively charged ristocetin tends to interact with the wall of the capillary. Increasing the phosphate buffer concentration lessens the wall interactions. However, there is a "trade-off", since increasing buffer concentration also increases heat generation and baseline noise and

Table 3
Effect of pH on the migration times, effective mobilities and resolution of enantiomers with ristocetin as a chiral selector

pH	Compound	R_s	$t(1)^a$	$t(2)^a$	$t(\mathrm{EOF})^b$	$\mu_e(1)^c$	$\mu_e(2)^c$
6	2-(3-Chlorophenoxy)-propionic acid	8.9	11.2	21.4	7.6	−3.1	−10.0
	Ketoprofen	5.7	12.3	14.3	7.5	−4.4	−6.2
	3-Methoxymandelic acid	1.5	16.8	20.2	7.6	−11.5	−13.0
	1-Benzocyclobutene-carboxylic acid	1.1	21.0	22.4	7.5	−13.2	−13.7
7	2-(3-Chlorophenoxy)-propionic acid	5.1	10.1	13.8	7.5	−4.9	−9.2
	Ketoprofen	2.6	12.6	13.7	7.5	−8.1	−9.1
	3-Methoxymandelic acid	1.1	18.1	20.2	7.4	−5.8	−6.7
	1-Benzocyclobutene-carboxylic acid	0.4	21.6	22.0	7.5	−13.4	−13.6

All separations were performed using 2mM ristocetin in 0.1 M phosphate buffer.
[a] The migration times of the enantiomers, $t(1)$ and $t(2)$, are given in minutes.
[b] The time corresponding to the electroosmotic flow, $t(\mathrm{EOF})$, is given in minutes. Note that a decrease in the EOF velocity results in an increase in $t(\mathrm{EOF})$.
[c] The effective mobilities, $\mu_e(1)$ and $\mu_e(2)$, are given in $\mathrm{cm^2\ kV^{-1}\ min^{-1}}$.

usually requires a decrease in the run voltage. The buffer concentration used throughout this work (i.e., 0.1 *M* phosphate, see Experimental) was a compromise after taking into account all of the aforementioned factors. A similar "wall-interaction effect" was noted previously for vancomycin [2]. The wall adsorption does not seem to affect the run-to-run reproducibility as long as all experimental conditions are held constant.

The effective mobilities (μ_e) of the analytes (which have electromigrations opposite to the direction of the electroosmotic flow) decrease with increasing concentration of ristocetin A (Table 2). This is because a greater amount of the analyte is associated with the ristocetin A when the concentration of the chiral selector is increased. Hence the improvement in the enantiomeric separations at higher ristocetin A concentrations seems to be the result of two factors: (1) a greater time of association between the chiral selector and analytes due to mass action and (2) a greater time of association between the chiral selector and analyte due to the decrease in the electroosmotic flow.

Table 3 shows the effect of pH on the enantioseparation of typical racemic analytes in this study. In general, lower pHs give better enantioresolution. However, there is a limit to this

Table 4
Effect of concentration of 2-propanol as an organic additive on the migration times, effective mobilities and resolution of enantiomers with ristocetin as chiral selector

Organic solvent	Compound	R_s	$t(1)^a$	$t(2)^a$	$t(\mathrm{EOF})^b$	$\mu_e(1)^c$	$\mu_e(2)^c$
None	2-(3-Chlorophenoxy)-propionic acid	5.1	10.1	13.8	7.5	−4.9	−9.2
	Ketoprofen	2.6	12.6	13.7	7.5	−8.1	−9.1
	3-Methoxymandelic acid	1.1	18.1	20.2	7.4	−5.8	−6.7
	1-Benzocyclobutene-carboxylic acid	0.4	21.6	22.0	7.5	−13.4	−13.6
10% 2-Propanol	2-(3-Chlorophenoxy)-propionic acid	5.8	14.6	21.3	11.5	−3.0	−6.5
	Ketoprofen	4.5	19.6	21.8	11.5	−5.8	−6.7
	3-Methoxymandelic acid	1.8	28.0	33.2	11.5	−8.3	−9.2
	1-Benzocyclobutene-carboxylic acid	0.7	40.2	40.6	11.5	−10.1	−10.1
20% 2-Propanol	2-(3-Chlorophenoxy)-propionic acid	7.6	21.4	30.8	11.8	−2.7	−6.2
	Ketoprofen	5.7	26.3	29.8	11.8	−7.6	−8.4
	3-Methoxymandelic acid	2.3	39.6	47.5	11.8	−9.7	−10.4
	1-Benzocyclobutene-carboxylic acid	1.1	55.0	57.9	11.7	−10.9	−11.0
30% 2-Propanol	2-(3-Chlorophenoxy)-propionic acid	10.0	26.6	47.7	14.5	−2.4	−5.1
	Ketoprofen	5.79	37.6	43.1	14.6	−4.2	−4.8
	3-Methoxymandelic acid	5.22	63.5	89.6	14.5	−6.0	−6.7
	1-Benzocyclobutene-carboxylic acid	1.23	70.1	73.7	14.6	−11.3	−11.6

All separations were performed using 2 m*M* ristocetin in 0.1 *M* phosphate buffer (pH7) with the indicated volume percentage of organic solvent.

[a] The migration times of the enantiomers, $t(1)$ and $t(2)$, are given in minutes.

[b] The time corresponding to the electroosmotic flow, $t(\mathrm{EOF})$, is given in minutes. Note that a decrease in the EOF velocity results in an increase in $t(\mathrm{EOF})$.

[c] The effective mobilities, $\mu_e(1)$ and $\mu_e(2)$, are given in $\mathrm{cm^2\ kV^{-1}\ min^{-1}}$.

effect as decreasing the pH will significantly decrease the electroosmotic flow (thereby increasing analysis times) and eventually protonate the analytes, making them neutral species. At sufficiently low pH, these two effects inhibit or negate the CE enantioseparation.

Data showing the effects of using an organic modifier in the running buffer are given in Table 4. As found with another macrocyclic antibiotic (rifamycin B), the use of organic modifiers can significantly alter the enantioresolution in some instances [1]. The addition of 2-propanol to the running buffer tended to enhance the enantioresolution for most analytes. Also, the organic modifier greatly increased the analysis (elution) times as a result of the decreased electroosmotic flow velocity. The effect of enhancing the enantioresolution by adding an organic modifier varies with the analyte. Eventually a level of organic modifier is reached that decreases the enantioresolution [1]. Also, when using aqueous–organic solvent mixtures in CE for longer periods of time, one must take precautions to prevent differential evaporation of the solution which will affect reproducibility, analysis times, resolution, etc.

Ristocetin A can decompose with time in aqueous solution. This process is accelerated at acidic or basic pHs and at elevated temperatures. For example, acid hydrolysis removes the sugars and an amine-containing fragment from the molecule [10]. Interestingly, the degradation product remains active against Gram-positive bacteria. However, the overall stability of ristocetin A under typical CE operating conditions (e.g., pH 5–7 and temperatures of 15–25°C) seems to exceed that of vancomycin [2]. We have used solutions of ristocetin A for up to 4 weeks provided that they are refrigerated (ca. 4°C) overnight or when otherwise not in use.

4. Conclusions

Ristocetin A is clearly a highly useful chiral selector for resolving a variety of enantiomers. Most of the racemic analytes in this study contained a free carboxylic acid moiety somewhere in their structure. Several experimental factors can be adjusted to optimize the enantioresolution and analyses times. These include the concentration of the chiral selector, the pH of the running buffer and the addition of moderate amounts of organic modifiers to the running buffer. Direct UV detection at wavelengths >250 nm is feasible because of the low concentrations of ristocetin A needed to achieve enantioresolution and its modest absorbance at those wavelengths. Electrostatic interactions between the racemic analyte and the chiral selector appear to be important in achieving enantioselective association. Extensive mechanistic studies on this and related systems are in progress.

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