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Enantiomeric resolution using the macrocyclic antibiotics rifamycin B and rifamycin SV as chiral selectors for capillary electrophoresis

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Abstract

Rifamycin B and rifamycin SV belong to the class of macrocyclic antibiotics known as ansamycins. These macrocyclic antibiotics were used as chiral selectors in capillary electrophoresis to enantioselectively resolve a number of chiral compounds. They contain groups capable of providing the types of multiple interactions necessary to achieve chiral recognition between enantiomers. In fact, they appear to be complimentary in the types of compounds they can enantiomerically resolve. Rifamycin B is shown to be enantioselective towards positively charged compounds, while rifamycin SV was enantioselective towards negatively charged solutes. The choice of wavelength for detection significantly affects sensitivity. Monitoring one of the wavelengths which coincide with the absorption minima of the chiral selector enhances sensitivity. Resolution is enhanced by keeping the amount of analyte injected on column as low as possible and it is demonstrated that it is possible to detect as little as 0.1% of one enantiomer in the presence of the other enantiomer using indirect detection.

1. Introduction

The separation of chiral compounds continues to be an area of significant interest, in part due to recent government regulations regarding the marketing of optically active drugs [1]. There are several approaches one can take to achieve enantiomeric separation using capillary electrophoresis (CE) [2–4]. Adding a chiral selector to the free solution is by far the most common approach [5,6], while immobilizing the chiral selector in a gel or other suitable packing in the capillary has also proven to be effective [7,8]. Other workers have demonstrated that wall-immobilized chiral stationary phases are feasible for enantiomeric separations [9,10].

The most common and successful additives for CE chiral separations have been the cyclodextrins and their derivatives [2,11]. This is due to the fact that cyclodextrins are of the 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 separation. In spite of their use, the need for more diverse and potentially more powerful chiral selectors still remains an intensive area of research. Recently Armstrong and

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co-workers suggested macrocyclic antibiotics would be useful as a broad new class of chiral selectors in CE, HPLC, and TLC [12–14]. These compounds which are structurally diverse and commercially available have opened a new area of research into chiral selectors that appear promising for enantioseparations.

Rifamycins have a characteristic ring structure or chromophore spanned by an aliphatic chain and differ from one another in the type and location of the substituents on their naphthohydroquinone ring. Fig. 1 shows how rifamycin B differs from rifamycin SV by the R group attached to the naphthohydroquinone ring. In rifamycin B this group is an oxy-acetic acid ($-OCH_2COOH$) while in rifamycin SV the R group is a hydroxyl (–OH). Changing this substituent has profound effects on the selector's selectivity toward charged compounds. Since the carboxylic and hydroxyl groups on rifamycin B are ionizable it can exist as a dibasic acid while rifamycin SV is essentially neutral at the pHs used in this study. In addition to the functional groups mentioned above, each rifamycin has nine stereogenic centers, four hydroxy groups, one carboxymethyl group, and one amide bond.

In this work the macrocyclic antibiotics rifamycin SV and rifamycin B were examined as chiral selectors for CE. These macrocyclic antibiotics appear to be complimentary in the types of compounds they can enantiomerically resolve. Rifamycin B seems to be well suited for separating positively charged analytes while rifamycin SV is capable of resolving negatively charged solutes. Previous work used rifamycin B as a chiral selector in CE for the resolution of a racemic amino alcohols [13]. They examined the effect of chiral selector concentration, pH, and organic modifier on enantiomeric resolution. In this work novel separations are presented using these chiral selectors and the effects of column loading and wavelength detection on sensitivity and separation are investigated.

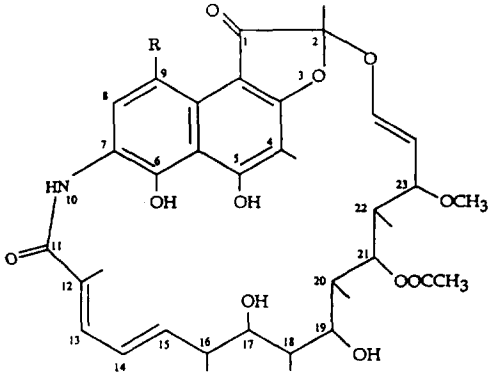


Fig. 1. Structures of rifamycin B (R = $-OCH_2COOH$) and rifamycin SV (R = –OH).

2. Experimental

Sodium dihydrogen phosphate, sodium hydroxide, and 2-propanol were obtained from Fisher (Atlanta, GA, USA); rifamycin B and rifamycin SV were acquired from Advanced Separation Technologies (Whippany, NJ, USA); and all chiral analytes were obtained from Sigma (St. Louis, MO, USA). All reagents in this study were used as received.

Electrophoretic experiments were performed using an Isco model 3850 (Isco, Lincoln, NE, USA) equipped with an on-column variable-wavelength UV detector, and a Chrom-Jet integrator was used to record electropherograms (Spectra-Physics, San Jose, CA, USA). The regulated high-voltage power supply capable of delivering up to 30 kV, was used in the constant voltage mode. An Ultra-Ware degassing system with helium gas supplied by Union Carbide (Danbury, CT, USA) was used to degas all solutions.

The fused-silica capillary tube was 65 cm × 50 μm I.D. (Isco), with the detector cell window 40 cm from the column inlet. All solutions were degassed and filtered prior to their use with 0.45-μm polypropylene filters (Alltech, Deerfield, IL, USA). The capillary was purged daily with 1.0 *M* NaOH, followed by water and run buffer for 3 min each. All samples, unless noted otherwise, were dissolved in distilled water at approximately 0.3 mg/ml and injected into the capillary using electrokinetic injection at 5 kV for 5 s. The capillary was purged with run buffer

for 3 min between injections. All separations were carried out at ambient temperature (21°C).

The aqueous buffer solutions were prepared by adjusting the pH of a solution containing the appropriate amount of sodium phosphate monobasic with NaOH. The phosphate buffer–2-propanol solvent mixtures are volume percents prior to mixing. Run buffers containing rifamycin B and rifamycin SV were prepared by weighing the proper amount of the macrocyclic antibiotic into a volumetric flask, adding the phosphate buffer–2-propanol solution, and sonicating to dissolve the antibiotic.

The absorbance spectra were obtained using a Perkin-Elmer Model 553 double beam UV–Vis spectrophotometer. The solution for the spectra was prepared in 70% 0.1 *M* phosphate buffer pH 7–30% 2-propanol, prepared as described above.

3. Results and discussion

3.1. Separations using rifamycin B as chiral selector

Recently, Armstrong and workers investigated the effect of pH, organic modifier and chiral selector concentration on the resolution of α-amino alcohols [13]. They found that enantioresolution increased with pH, reached a maximum at pH 7, and decreased with increasing pH. This can be explained by examining the charge on the chiral selector and analyte being separated. Rifamycin B, a dibasic acid, loses some fraction of the negative charge present on the molecule as pH is lowered which precludes a strong charge–charge interaction with the positively charged amine containing analyte. As pH is increased to pH 7, rifamycin B exists primarily as a di-anion while the analyte is positively charged, providing a strong electrostatic interaction. At higher pHs, the amine group on the analyte is deprotonated again precluding a strong charge–charge interaction. While other parameters such as electroosmotic flow are also affected, clearly charge–charge interactions play a prominent role in enantioresolution.

No enantioselectivity was observed in the absence of organic modifier in the run buffer. Of the organic modifiers investigated, 2-propanol provided the greatest enhancement to enantioresolution. Increasing the 2-propanol concentration in the run buffer increased enantioresolution, decreased electrophoretic mobilities and increased migration times. Increasing chiral selector concentration increased enantioresolution but tended to slightly increase migration times due to the small decrease in electrophoretic mobility. Increasing the chiral selector concentrations above 30 m*M* produced an extremely small signal-to-noise ratio due to the strong UV absorption of the chiral selector.

Considering all the factors discussed above, we chose a buffer containing 25 m*M* rifamycin B in 70% 0.1 *M* phosphate buffer pH 7–30% 2-propanol for our studies.

Influence of wavelength on sensitivity

Rifamycin SV and rifamycin B absorb strongly in both the ultraviolet and visible spectral regions as shown in Fig. 2. This makes direct detection of analytes difficult at commonly employed wavelengths such as 254 nm. Also in Fig. 2, it can be seen that rifamycin SV has absorption maxima at slightly longer wavelengths than rifamycin B, but each exhibit minima at approximately 205, 275 and 350 nm. A series of racemic

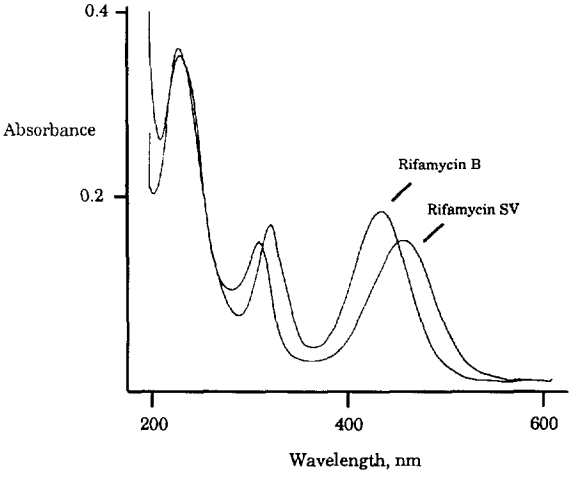


Fig. 2. UV–Vis spectra of rifamycin B and rifamycin SV at pH 7.

amino alcohols were previously resolved using a wavelength of 254 nm by indirect detection [13]. In this work we examined the effect of wavelength detection on sensitivity, specifically looking at regions where an absorption minima occurred. Fig. 3 shows the separation of epinephrine injected under identical conditions at 275 nm and 350 nm. It is apparent from Fig. 3 that the sensitivity is much greater at 350 nm than at 275 nm. This can be attributed to the fact that at absorption minima the baseline noise is substantially reduced resulting in improved sensitivity. Since the sensitivity was greater at 350 nm than at 275 nm or 254 nm, we chose this wavelength for all subsequent injections.

Effect of column loading on resolution

Table 1 shows the results of injecting seven compounds most of which had resolutions factors (R_s) of 1.0 or less from the previous work [13]. In that work the authors found that a concentration of 25 m*M* rifamycin B in 60% 0.1 *M* phosphate buffer pH 7–40% 2-propanol afforded the best enantioselectivity. We used the same buffer system with the exception of adding 30% 2-propanol instead of 40% 2-propanol. Using a larger percent of 2-propanol increases resolution

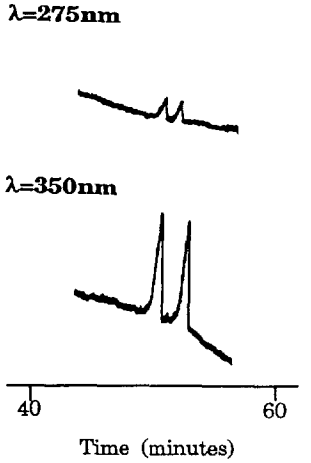


Fig. 3. CE separation of enantiomers of epinephrine at 275 nm and 350 nm. Run buffer consisted of 2-propanol–0.1 *M* phosphate buffer pH 7 (30:70, v/v) containing 25 m*M* rifamycin B. Separation voltage was 8 kV and 1.0 mg/ml of epinephrine was loaded at 5 kV for 5 s.

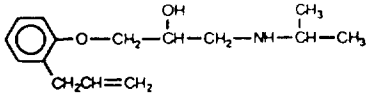
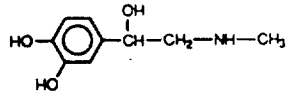
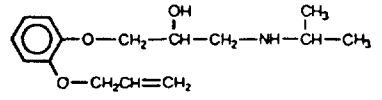
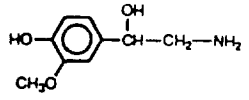
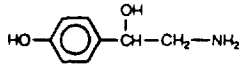
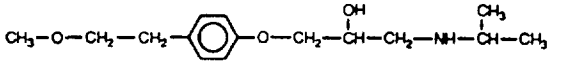
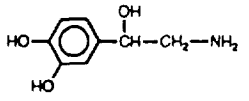
but also substantially increases solute migration times. As a compromise we chose 30% 2-propanol since enantioresolutions at this percent organic modifier were good and migration times were substantially shortened. By injecting approximately 0.2 to 0.3 mg/ml at 5 kV, 5 s, we were able to triple the R_s value of oxprenolol (0.4 to 1.2), double the values for alprenolol (0.7 to 1.4) and epinephrine (1.5 to 3.1), and substantially improve the values for normetanephrine (0.9 to 1.3) and octapamine (1.1 to 1.4). It should be noted from Table 1 that most of these separations could be improved further by increasing the amount of 2-propanol at the expense of longer analysis time. Interestingly, reducing the amount of metaprolol and norepinephrine loaded on column appeared to have little effect on their resolution. Resolution and peak-to-peak separation are affected by the amount of analyte loaded on the capillary column. With the increase in sensitivity at 350 nm (greatest UV minima), loading less analyte (approximately 80% less) on column substantially improved separation resolutions for five of the seven solutes studied.

The amount of analyte loaded on column clearly affects chiral resolution as noted above. The effect on resolution as well as peak shape is shown in Fig. 4. As more epinephrine was loaded on column, separation gradually eroded. From the figure it is apparent that the separation began degrading significantly between 0.3 mg/ml and 60 mg/ml and is nearly lost at 200 mg/ml. As the concentration loaded increased above 100 mg/ml, peak tailing became very pronounced and resolution began to degrade rapidly. Thus separations of amounts larger than what could be termed analytical, are not feasible with this system due to the loss of resolution as well as poor peak symmetry at higher loadings of analyte on column.

Separation of compounds containing more than one ring

Table 2 shows the first separation of several new solutes using either rifamycin B or rifamycin SV as indicated in the table. The first three solutes were all positively charged under the

Table 1
Increased enantiomeric resolutions of selected compounds, migration times and apparent mobilities with 25 m*M* rifamycin B in 2-propanol–0.1 *M* phosphate buffer pH 7.0 (30:70, v/v)

Compound	R_s	Time[a]	μ(a)[b]	R_s[c]	Time	μ(a)
Alprenolol 	1.4	19.2	16.9	0.7	63.9	5.6
Epinephrine 	3.1	48.2	6.7	1.5	62.1	5.8
Oxprenolol 	1.2	35.8	9.1	0.4	60.3	6.0
Normetanephrine 	1.3	29.2	11.1	0.9	55.6	6.5
Octapamine 	1.4	37.6	8.6	1.1	55.6	6.5
Metaprolol 	0.7	46.1	7.0	0.8	62.1	5.8
Norepinephrine 	0.9	32.8	9.9	0.9	57.0	6.3

[a] Migration times are given in minutes for first eluting isomer.
[b] μ(a) is the apparent mobility in $cm^2\ kV^{-1}\ min^{-1}$ of the first eluting isomer.
[c] Data from Ref. [13].

conditions employed, while the last three solutes were negatively charged. Rifamycin B had previously been shown to be adept at separating single-ring structures but appeared to exhibit no enantioselectivity towards double-ring or larger structures. Pindolol and propranolol were both resolved even though both contain multiple-ring structures. In the case of pindolol, it is composed of an indole ring which is substituted at the 4 position with an aliphatic chain containing an amine. It exhibits an absorption profile in the UV range similar to that of the previous single-ring aromatic structures monitored by indirect detection, in that it does not absorb appreciably at 350 nm. Propranolol on the other hand contains a naphthyl moiety which absorbs UV light significantly at longer wavelengths and was monitored by direct detection at 350 nm. While pindolol was poorly resolved, propranolol exhibited good resolution. It is of interest to note

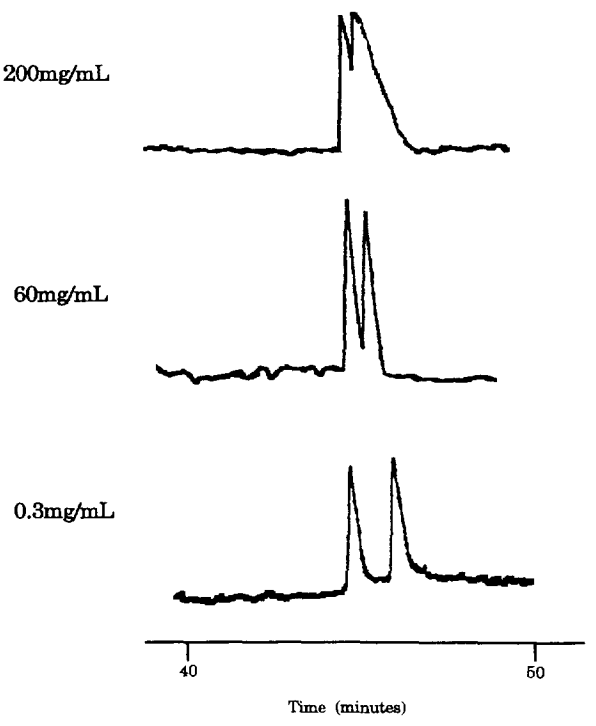


Fig. 4. The effect of solute concentration versus resolution at 350 nm using indirect detection. Conditions: same as Fig. 3. Separation voltage was 8 kV and indicated concentration of epinephrine was loaded at 5 kV for 5 s.

that the two compounds contain one identically substituted ring and differ only in that pindolol is 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.

3.2. Separations using rifamycin SV as the chiral selector

The last three solutes in Table 2 were resolved using rifamycin SV as the chiral selector in the run buffer. All three were negatively charged at the buffer pH employed, and eluted after the larger water perturbation of the baseline. Fig. 5 shows the separation of hexobarbital using indirect UV detection. Interestingly, rifamycin SV seems particularly suited for separating systems containing at least two rings. Hexobarbital and glutethimide each contain two rings which are not conjugated while dansyl aspartic acid has a conjugated ring system. Dansyl aspartic acid absorbs at longer wavelengths, and was monitored by direct UV detection at 350 nm while hexobarbital and glutethimide were both monitored by indirect UV detection. Rifamycin SV separates negatively charged solutes and is a complimentary chiral selector to rifamycin B, which can resolve positively charged solutes.

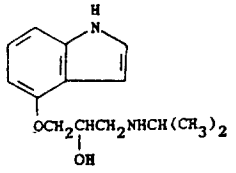
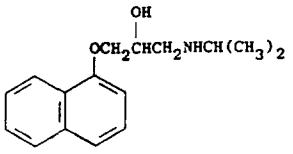
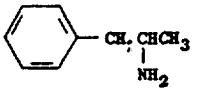
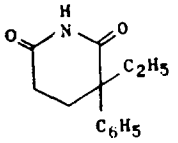
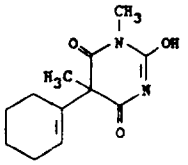
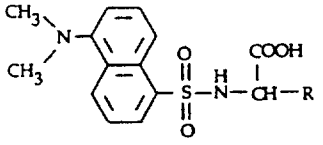
3.3. Enantiomeric purity assessment

The ability to monitor one enantiomer in the presence of the other remains an important consideration for any viable chiral analysis. Fig. 6 shows the separation of isoproterenol with spiked concentrations of each enantiomer. Using indirect UV detection at 350 nm we could accurately see 0.1% of the (−)-enantiomer in the presence of the (+)-enantiomer. In this case the enantiomer as the minor constituent eluted first, which makes monitoring optical purity easier.

4. Conclusions

We have demonstrated the utility of rifamycin B and rifamycin SV in the chiral resolution of several compounds. We found that performing separations at 350 nm versus 275 or 254 nm results in increased sensitivity due to the decrease in baseline noise at longer wavelengths. This increase in signal-to-noise ratio allows for smaller amounts of solute to be injected on column which can increase chiral resolution for some compounds. While solutes containing single aromatic groups are especially suited for chiral resolution using rifamycin B, solutes containing more than one ring are also amenable to separation. Rifamycin SV resolved negatively charged solutes making it a complimentary chiral selector to rifamycin B which resolves positively charged solutes. It was shown that it is possible to measure as little as 0.1% of one enantiomer in the presence of the other demonstrating the feasibility of using these chiral selectors for

Table 2
Enantiomeric resolutions, migration times and apparent mobilities with 25 m*M* macrocyclic antibiotic in 2-propanol–0.1 *M* phosphate buffer pH 7.0 (30:70, v/v)

Compound	R_s	Time[a]	μ(a)[b]
Pindolol[c]	0.3	36.7	8.8
			
Propranolol[c]	1.3	27.8	11.7
			
Amphetamine sulfate[c]	1.1	36.1	9.0
			
Glutethimide[d]	4.0	34.8	9.3
			
Hexobarbital[d]	1.9	40.8	8.0
			
Dansyl aspartic acid[d]	1.5	36.8	8.8
			

[a] Migration times are given in minutes for first eluting isomer.
[b] μ(a) is the apparent mobility in $cm^2\ kV^{-1}\ min^{-1}$ of the first eluting isomer.
[c] Positively charged compounds resolved with rifamycin B.
[d] Negatively charged compounds resolved with rifamycin SV.

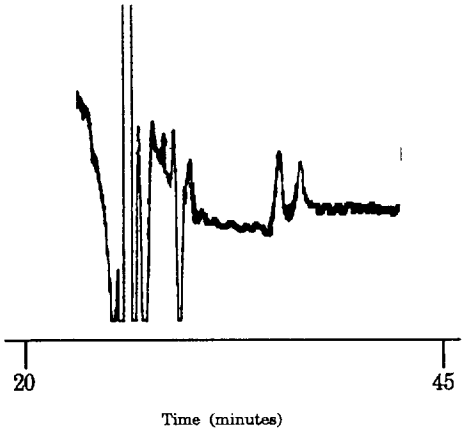


Fig. 5. Separation of enantiomers of hexobarbital. Conditions: run buffer, 2-propanol–0.1 *M* phosphate buffer pH 7 (30:70, v/v) containing 25 m*M* rifamycin SV. Voltage was 8 kV, indirect detection at 350 nm.

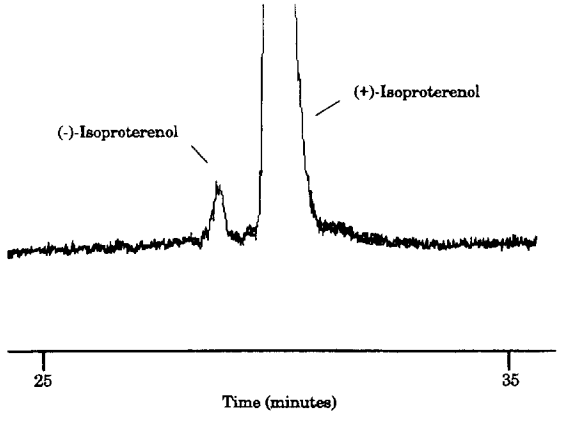


Fig. 6. Separation of enantiomers of isoproterenol at concentrations of 500 μg/ml (+)-isoproterenol and 0.50 μg/ml (−)-isoproterenol using indirect UV detection at 350 nm. Injection was at 5 kV for 8 s. Other conditions same as Fig. 3.

enantiomeric purity assay. Increasing the amount of solute loaded on column rapidly degrades separation, thus for best results, solute loadings should be kept as low as feasible for detection.

Work is continuing in our laboratory to further characterize the parameters which affect separation and investigation of the mechanism of separation for these macrocyclic chiral selectors.

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