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Optimization strategies for HPLC enantioseparation of racemic drugs using polysaccharides and macrocyclic glycopeptide antibiotic chiral stationary phases

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Abstract

The chiral resolution by high performance liquid chromatography (HPLC) is controlled by a number of parameters. The optimization of HPLC parameters is an important issue in chiral resolution. This review discusses the optimization of HPLC conditions for the chiral resolution of racemic drugs on polysaccharides and macrocyclic glycopeptide antibiotic chiral stationary phases (CSPs). The most important parameters discussed are composition of mobile phase, pH of mobile phase, flow rate, temperature and effect of other parameters.

Keywords: Optimization; HPLC conditions; Enantiomeric resolution; Polysaccharides CSPs; Macrocyclic glycopeptide antibiotic CSPs

1. Introduction

One of the two enantiomers of a drug may be toxic or sometimes inactive with respective to biological systems [1]. The differences in the biological activities of the enantiomers are due to the differences in protein binding and transport, mechanism of action, rate of metabolism, changes in activities due to metabolism, clearance rate and persistence in the environment [2–4]. Because of the different biological activities of enantiomers of active ingredients, the preparation of highly enantio-pure compounds is of utmost importance [5,6]. Therefore, the chiral resolution is essential in pharmaceutical, agriculture and food industries. In view of this, US Food and Drug Administration has issued certain guidelines for the marketing of racemic compounds [7]. In last two decades, high performance liquid chromatography (HPLC) has become one of the mostly applied modality in the chiral resolution of different racemates [8,9]. Several chiral stationary phases (CSPs) have been developed and used for the chiral resolution of a variety of racemates. The important CSPs include Pirkle types [10,11], derivatized linear or helical (cellulose or amylose) polysaccharides [5,6,12], cyclodextrin and its derivatives [13–15], protein phases [16], chiral crown ethers [17–19], macrocyclic glycopeptide antibiotics [13,16,18–20] and ligand exchange [21]. Among these CSPs, polysaccharides and macrocyclic glycopeptide antibiotic based CSPs are very important as they have achieved a great reputation in the field of chiral resolution. The importance of these two types of CSPs include their ease of use, reproducible results and a wide range of applications [8,9,13,16,20–24]. The enantiomeric resolution is very sensitive and is controlled by a number of HPLC parameters. Therefore, the control and the optimization of HPLC parameters are very important issue in enantiomeric resolution by HPLC. In view of this, attempts have been made to discuss the optimization of HPLC conditions for enantiomeric resolution on polysaccharides and macrocyclic glycopeptide antibiotic CSPs.

2. Optimization of HPLC conditions

There are many parameters which control the enantiomeric resolution by HPLC but the most important

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include composition and pH of mobile phase, flow rate of mobile phase and temperature. Before the discussion of the art of the optimization of HPLC conditions it is essential to have the knowledge of the structures of the CSPs and the possible mechanisms of chiral resolution. Therefore, a brief outline on the structures of polysaccharides and macrocyclic glycopeptide antibiotic CSPs and their mode of mechanism is presented herein.

Among the various polysaccharides polymers such as cellulose, amylose, chitosan, xylan, curdlan, dextran and insulin, cellulose and amylose have been used for the preparation of commercial CSPs [11]. Cellulose and amylose itself could not used as commercial CSPs because of their poor resolution capacity and problem in handling [12]. Therefore, these polymers have been derivatized as their tricarbamate or triesters [9,11,16]. The polymeric chains of D−(+) glucose units contains β-1,4 linkage in cellulose and α-1,4 linkage in amylose, respectively. These chains lie side by side in a linear fashion in cellulose and in helical fashion in amylose (Fig. 1). Polysaccharides based CSPs are available in normal and reversed phase modes. About 20 derivatives of cellulose and amylose, as shown in Fig. 2, are commercially available from Daicel Chemical Industries, Japan. The trade name of the cellulose and amylose derivatives are Chiralcel and Chiralpak, respectively. To denote the reversed phase nature of the CSPs, R is added in the last of Chiralcel and Chiralpak trade names. The chiral recognition mechanism at a molecular level on the polysaccharides based CSPs is still unclear although it has been reported that the chiral resolution by these CSPs is achieved through the different hydrogen, π–π and dipole–dipole induced interactions between the CSP and the enantiomers [25–27]. Recently, it has been observed that coordination bonding is also contributing in the chiral resolution of sulfur containing enantiomers [28]. In addition to these bondings, steric effect also governs the chiral resolution on polysaccharides CSPs [25,29]. Therefore, the three dimensional structure of the racemates (with functional group and aromatic rings) fit stereogenically in the different fashion into the chiral grooves of the stationary phases which is stabilized by these bondings at different magnitude for both (+) and (−) enantiomers and hence the resolution of enantiomers occurred.

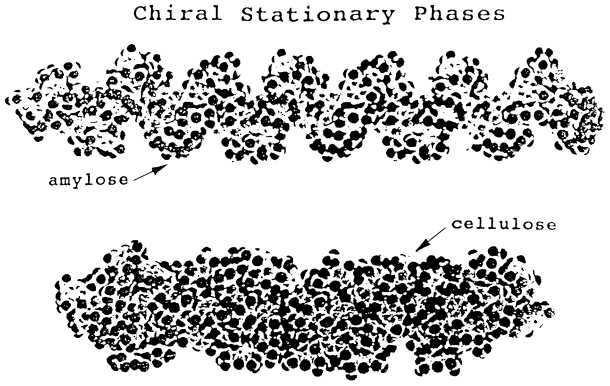


Fig. 1. Three dimensional structures of amylose and cellulose.

By scanning through the literature, it was found that only nine antibiotics have been used as the chiral selectors for the enatiomeric resolution of a variety of racemic drugs. The reported antibiotics include vancomycin, teicoplanin, teicoplanin aglycon, thiostrepton, rifamycin B, kanamycin, streptomycin, fradiomycin and ristocetin A. It is very interesting to note that all of them contain ionizable groups at different pH values in their structures. The structures of these antibiotics are shown in Fig. 3. The complex structures (three-dimensional structures and different spatial stereochemical arrangements of the functional groups) of the antibiotics containing different chiral centers, inclusion cavities, phenyl rings, pyranose, furanose, quinoline and thiazole rings, several hydrogen donor and acceptor sites, sugar moieties, and other groups which are responsible for their surprising chiral selectivities in different modes. This allows for an excellent potential to resolve a greater variety of racemates. The possible interactions involved with the use of antibiotics as chiral selectors for chiral recognition are π–π complexation, hydrogen bonding, inclusion complexation, dipole interactions, steric interactions, anionic and cationic bindings [19]. These interactions take place individually or in combinations, which can result in the very high chiral recognition capacities for these antibiotics. The strength of these interactions depends upon the type of phases used. The reversed phase condition favors the inclusion and hydrogen bonding. On the other hand, the normal phase favors the π–π complexation and dipole interactions. The new polar organic phase mode enhances all of the above interactions. The commercial macrocyclic glycopeptide antibiotic CSPs are available from Astec, Whippaney, NJ, USA. The most commonly available CSPs are Chirobiotic V, Chirobiotic R, Chirobiotic T and Chirobiotic TAG, which corresponds to vancomycin, ristocetin, teicoplanin and teicoplanin aglycon antibiotics, respectively.

3. Optimization of HPLC conditions of enantiomeric resolution on polysaccharides based CSPs

The chiral resolution is sensitive on polysaccharides based CSPs and, therefore, the optimization of HPLC conditions on these phases is very important issue. The most important factors, which control enantiomeric resolution, are composition of mobile phase, pH of mobile phase, flow rate of the mobile phase, temperature and other parameters. The optimization of these parameters on polysaccharides based CSPs is discussed below.

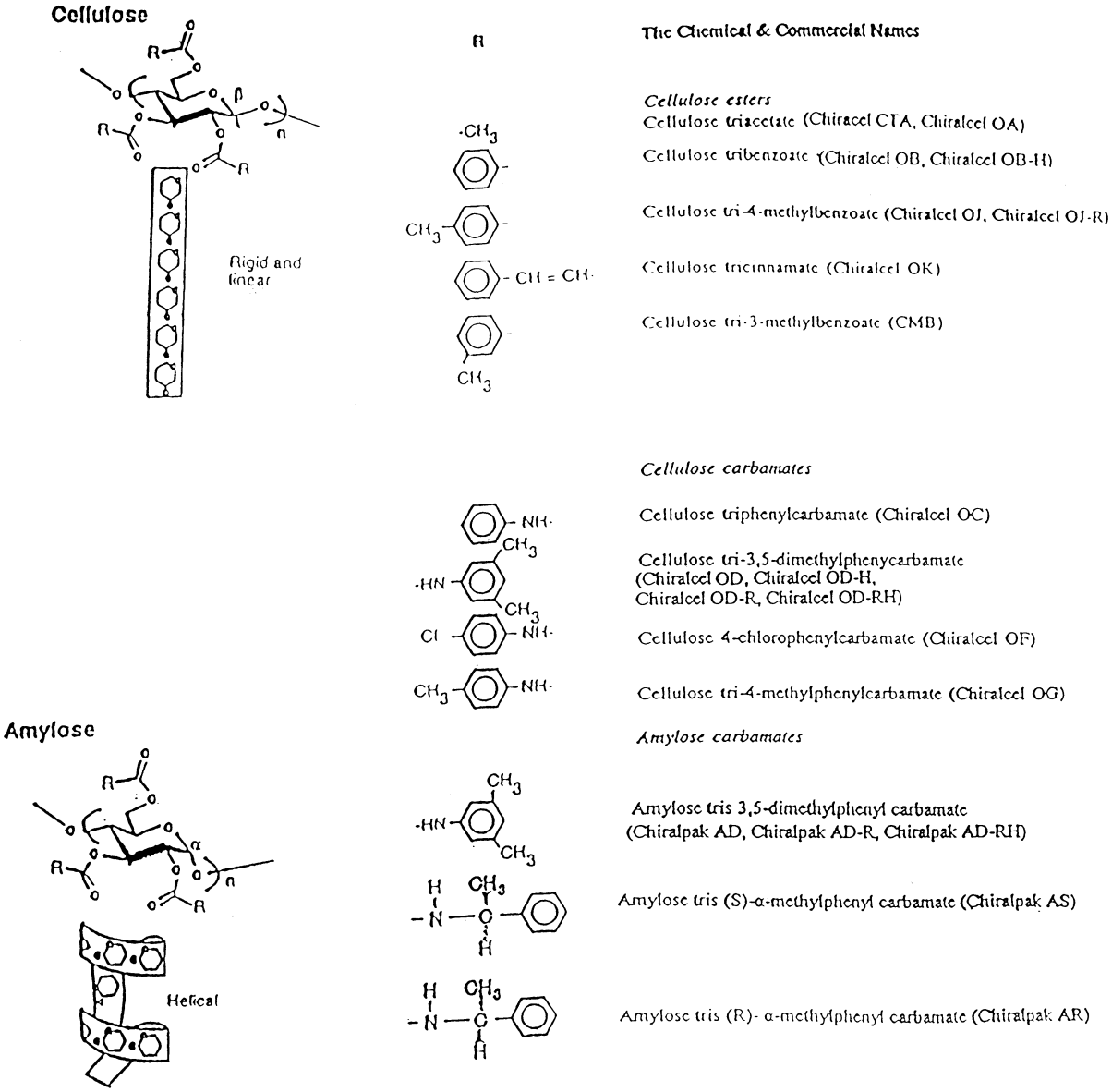


Fig. 2. The chemical structures, chemical and trade names of most commonly used polysaccharides based CSPs.

3.1. Mobile phase compositions

The selection of mobile phase is the key aspect in chiral resolution. The mobile phase is selected according to the solubility and the structure of the drugs to be resolved. In normal phase mode mostly hexane, cyclohexane, pentane and heptane are used as the major constituent of the mobile phase. However, other solvents like alcohols, acetonitrile are also used in the mobile phase. Normally, hexane, 2-propanol or ethanol in the ratio of 80:20 is used as the mobile phase and the change in mobile phase composition is carried out based on the observations. Finally, the optimization of chiral resolution is carried out by adding the little amount of amines or acids (0.1–1.0%). The protocol of the selection and optimization of mobile phases for the enantiomeric resolution of drugs on polysaccharides based CSPs in normal phase mode is presented in Scheme 1. The effect of isopropanol on the chiral resolution of certain drugs has been studied by Wainer and Stiffin [26] and is given in Fig. 4. The effect of mobile phase composition was also studied by Bonato et al. [30] for the enantiomeric resolution of propafenone, 5-hydroxypropafenone and *N*-despropylpropafenone on Chiralpak AD column. Recently, Aboul-Enein and Ali [31] have observed the reverse order of elution of nebivolol enantiomers on Chiralpak AD CSP when using ethanol and 2-propanol separately as the mobile phases. However, the best resolution was obtained when using ethanol as the mobile phase.

The chiral resolution on polysaccharides based CSPs in reversed phase mode is carried out by using aqueous

mobile phases. Again the selection of mobile phase depends on the solubility and the properties of the drugs to be analyzed. The choice of the mobile phase under reversed phase mode is very limited. Water is used as the main constituent of the mobile phases. The modifiers used are acetonitrile, methanol, ethanol. The optimization of chiral resolution is carried out by adding small percentage of amines or acids (0.1–1.0%). Some of the resolutions are pH dependent and require the constant pH of the mobile phase. Under such conditions, generally, the resolution is not reproducible when using water–acetonitrile or –methanol etc. mobile phases and, therefore, buffers with some organic modifiers (acetonitrile, methanol etc.) have been used as the mobile phase. The optimization of resolution is carried out by adjusting the pH of the buffers and the amount of organic modifiers. The most commonly used buffers are perchlorate, acetate and phosphate buffers. The protocol of the selection and optimization of mobile phase for the enantiomeric resolution of drugs on polysaccharides based CSPs in reversed phase mode is presented in Scheme 2. The correlation of separation conditions of neutral, acidic and basic drugs on polysaccharides based CSPs are presented in Table 1.

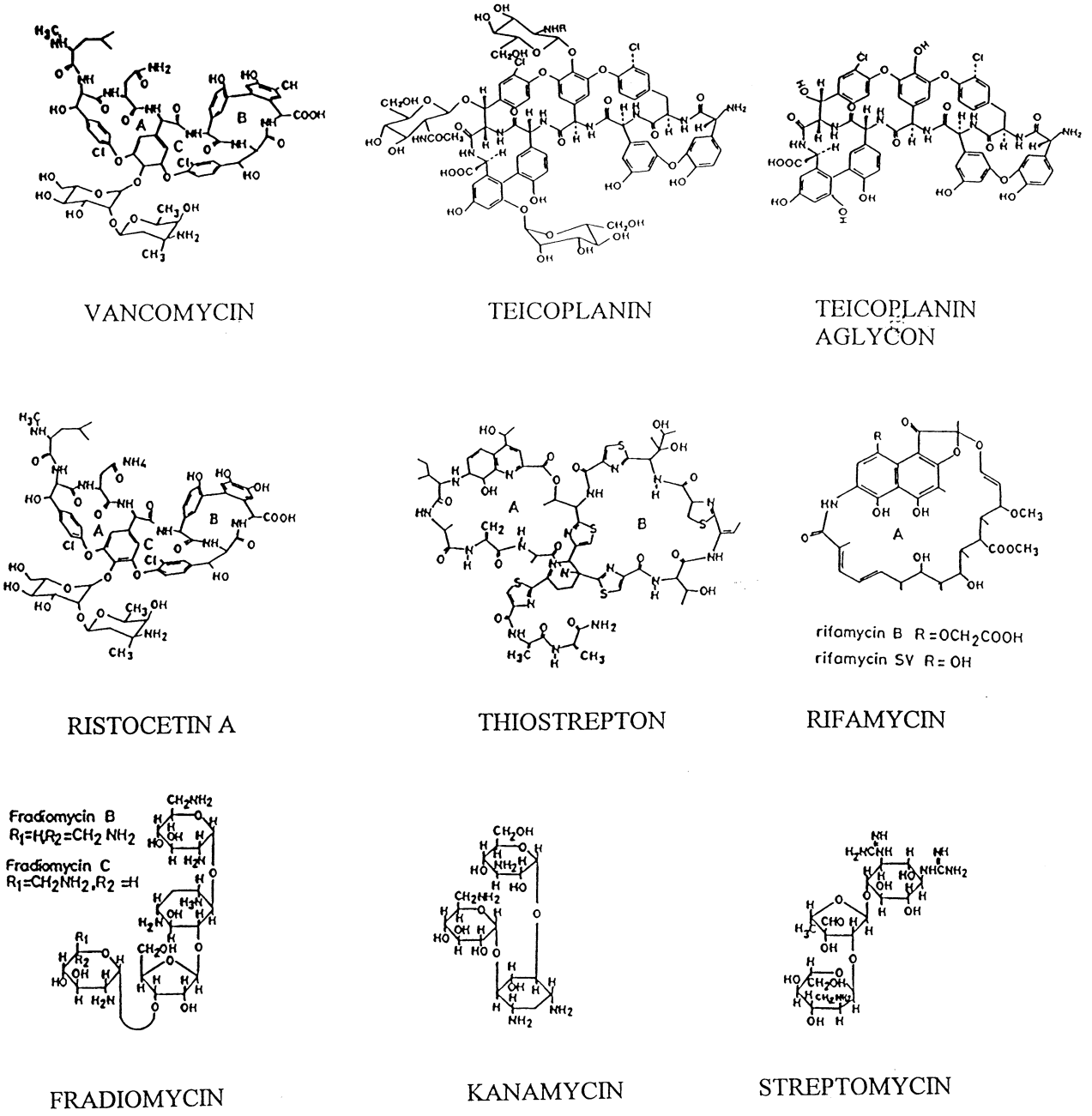
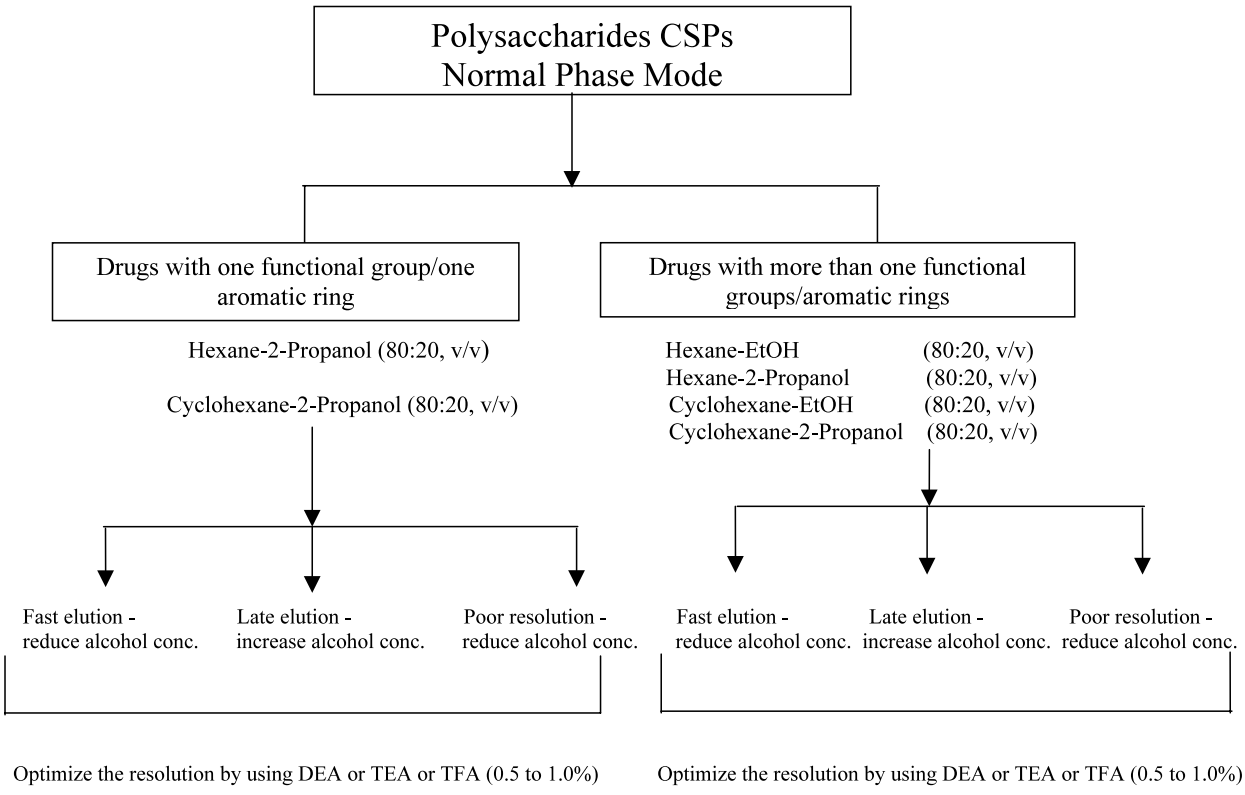


Fig. 3. The chemical structures of antibiotics used in the preparation of antibiotic based CSPs.



Note: This is the brief outline of the procedure to follow in developing a resolution on polysaccharides CSPs on normal phase mode. However, other mobile phases may be used.

Scheme 1. The protocol for the development and optimization of mobile phases on polysaccharides based CSPs on normal phase mode.

The resolution was effected by changing the polarity of the mobile phases. It is very interesting to note that the change in resolution with respect to the mobile phase compositions varied from compound to compound. The resolution on polysaccharides based CSPs in reversed phase mode have been improved by adding the cations and anions. The order of retention, of propranolol enantiomers on Chiralcel OD-R using sodium perchlorate salt–acetonitrile (60:40, v/v) as mobile phase, in the presence of cations was $Na^+ > Li^+ > K^+ > NH_4^+ > N(C_2H_5)_4^+$ while this order in the presence of anions was $ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^- > AcO^-$ [32]. The effect of acetonitrile concentration on the resolution of *R*,*S*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrazine-4-spiro-3′-pyrrolidine)-1,2′,3,5′-tetrone has been carried out by Kazusaki et al. [33] and the results are given in Fig. 5. It has been reported that the resolution factor decreased with an increase of acetonitrile concentration. Aboul-Enein and Ali [34] have studied the effect of acetonitrile content on the chiral resolution of flubiprofen on Chiralpak AD-RH column. Fig. 6 indicates the

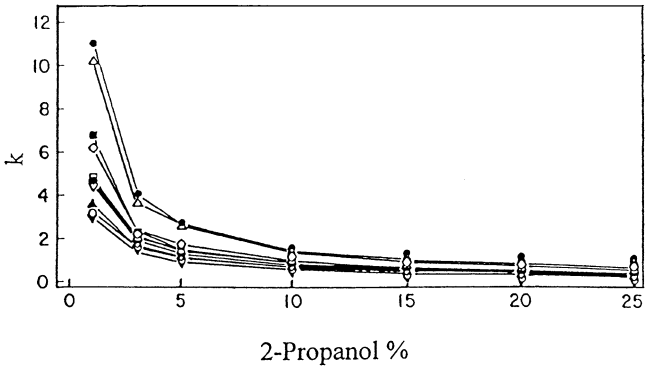
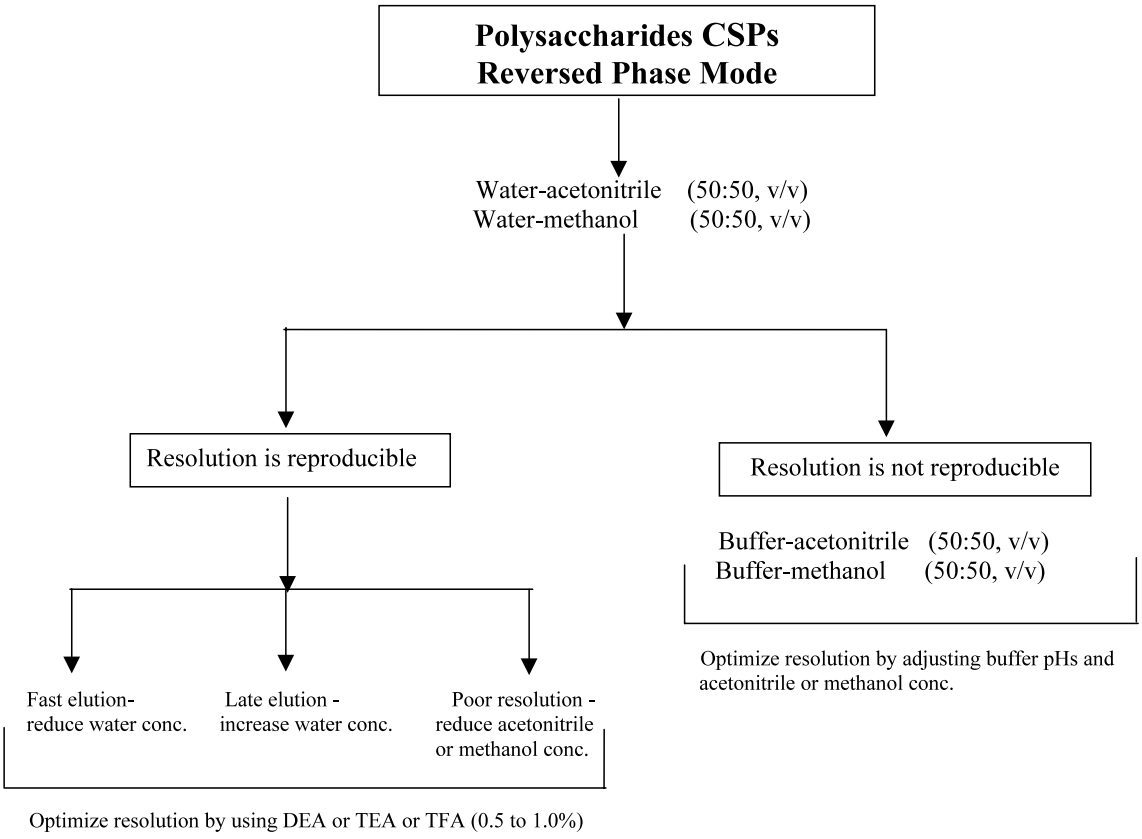


Fig. 4. The effect of the concentration of 2-propanol in the mobile phase hexane-2-propanol. Second eluted isomer solute 1 (△), first eluted isomer solute 1 (◇), second eluted isomers solute 2 (◆), first eluted isomer solute 2 (▲), solute 3 (○), second eluted isomer solute 4 (□), first eluted isomer solute 4 (▼), solute 5 (●), solute 6 (■) and solute 7 (▼). Solutes 1: 1-phenylethanol, solute 2: 1-phenylpropanol, solute 3: 1-phenyl propanol-2, solute 4: 2-phenylpropanol, solute 5: benzylalcohol, solute 6: 3-phenyl propanol-1, solute 7: 2-phenyl propanol-2 [26].



Note: This is the brief outline of the procedure to follow in developing a resolution on polysaccharides based CSPs on reversed phase mode. However, other mobile phases may be used.

Scheme 2. The protocol for the development and optimization of mobile phases on polysaccharides based CSPs on reversed phase mode.

Table 1
The correlation of separation conditions of neutral, acidic and basic compounds

Comp.	Systems	
	Normal phase	Reversed phase [a]
Neutral	MP = IPA/hexane, pH has no effect on the resolution	MP = water/ACN, pH has no effect on the resolution
Acidic	MP = IPA/hexane/TFA, pH near 2.0	MP = pH 2.0 perchlorate, acid/CAN
Basic	MP = IPA/hexane/DEA, IPA/hexane/TFA with pH near 2.0, ion-pair separation	MP = buffer/CAN, typical buffer is 0.5 M $NaClO_4$ with pH in range of 4.0–4.5, ion-pair separation

MP, mobile phase; IPA, isopropanol; ACN, acetonitrile; TFA, trifluoroacetic acid; and DEA, diethylamine.
[a] Columns are normally not run under basic conditions.

pattern of flubiprofen resolution with different percentages of acetonitrile. Again, high concentration of acetonitrile resulted in poor resolution.

3.2. pH of the mobile phase

pH also controls the chiral resolution of different racemic drugs on polysaccharides based CSPs. Aboul-Enein and Ali [28,35] have observed that the chiral resolution on polysaccharides based CSPs is pH dependent under normal phase mode. They have observed the partial resolution of certain antifungal agents at lower pH while the resolution was improved by increasing the pH with triethylamine on amylose and cellulose chiral columns. Further, Aboul-Enein and Ali [34] have studied the effect of pH on the chiral resolution of

flubiprofen on Chiralpak AD-RH column. Fig. 7 indicates that pH 3.5 as the best pH for the resolution of flubiprofen. In reversed phase mode also the chiral resolution on polysaccharides CSPs are pH dependants. Therefore, buffers have been used to achieve the best resolution. Generally, lower pH values of the buffers have been recommended for the best resolution.

3.3. Flow rate

The chiral resolution can be controlled by flow rate on polysaccharides CSPs. However, there are only few studies dealing with the optimization of chiral resolution by adjusting flow rates. Aboul-Enein and Ali [28] have optimized the chiral resolution of some antifungal agents on Chiralpak AD, AS and AR CSPs by adjusting the flow rates. The flow rates were varied from 0.5 to 2.0 ml/min but the best resolution could be achieved at 0.5 ml/min flow rate. Table 2 shows α and Rs values of the antifungal agents at 0.5 and 1.0 ml/min flow rates. Table 2 also indicates no resolution of miconazole and sulconazole and a partial resolution of econazole was achieved using 1 ml/min flow rate. Therefore, 0.5 ml/min flow rate was found suitable for this study. It is interesting to note that there was no improved resolution by decreasing the flow rate below 0.5 ml/min.

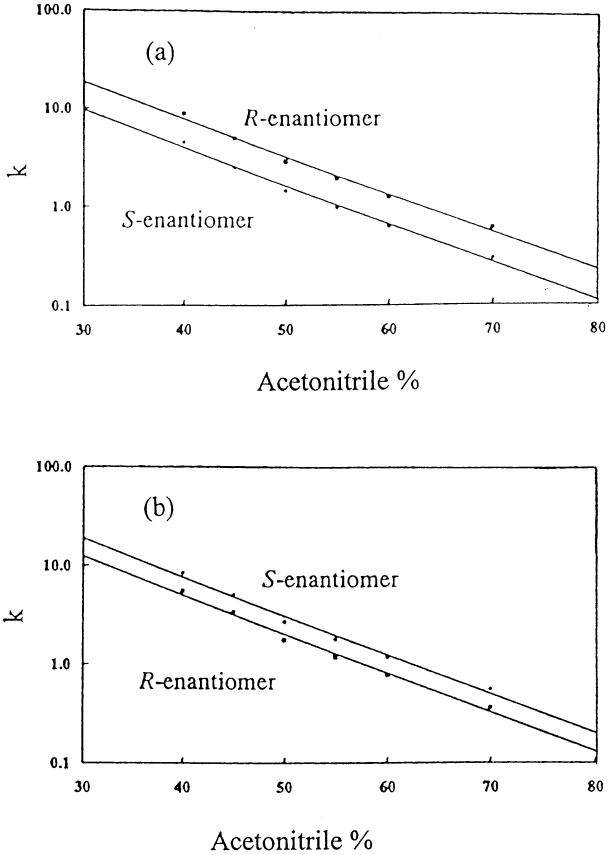


Fig. 5. Retention of enantiomers of *R,S*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4 - tetrahydropyrrolo[1,2 - *a*]pyrazine - 4 - spiro - 3′ - pyrrolidine)-1,2′,3,5′-tetrone on: (a) Chiralpak AD-RH; (b) Chiralcel OD-RH columns using a mixture of acetonitrile and 0.01 M acetate buffer (pH 4.7) [33].

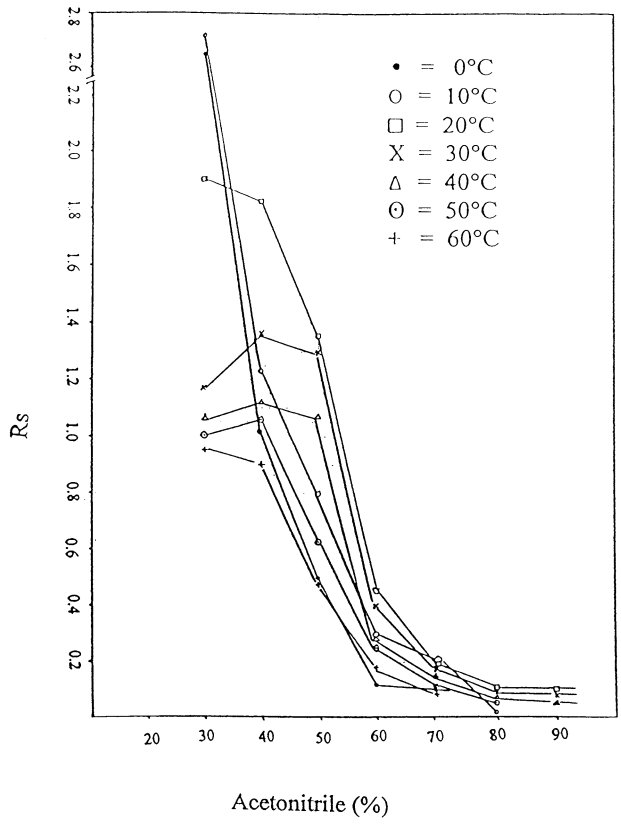


Fig. 6. The relationship between Rs and percentages of acetonitrile at different temperatures.

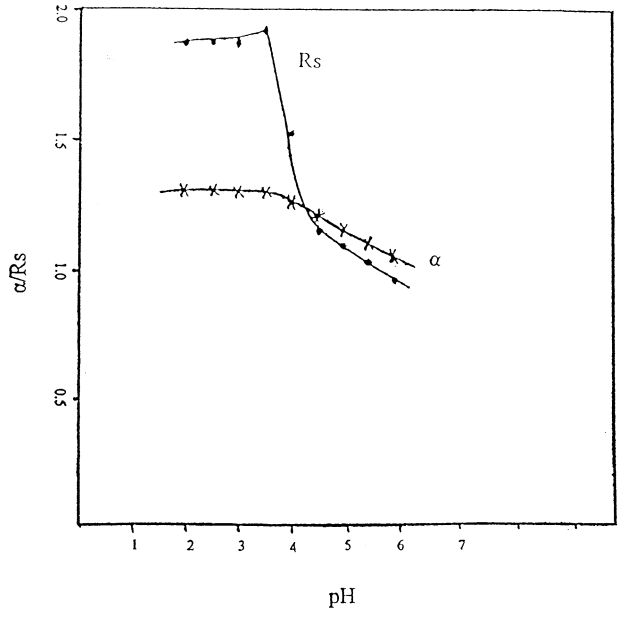


Fig. 7. The relationship between α/Rs and pH of the mobile phase water–acetonitrile (60:40, v/v).

3.4. Temperature

Temperature is also contributing to the chiral resolution of racemic drugs on polysaccharides CSPs. Only a few studies are available which deal with the influence of temperature on chiral resolution. The effect of temperature on the capacity factor of nebivolol enantiomers on Chiralpak AD is given in Fig. 8 [23] indicating the poor resolution at higher temperature. Furthermore, the resolution of benzatriazole derivatives on Chiralcel OJ, as a function of temperature, is given in Fig. 9 [23] which again shows the decrease in resolution at higher temperature. The effect of temperature on the chiral resolution of *R,S*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrazine-4-spiro-3′-pyrrolidine)-1,2′,3,5′-tetrone on Chiralpak AD-RH column was studied by Kazusaki et al. [36] and the results are shown in Fig. 10. The chiral resolution of flubiprofen on Chiralpak AD-RH column has been studied by Aboul-Enein and Ali [34]. They have observed that the best resolution occurred at 25 °C.

Table 2
The effect of flow rate on chiral resolution of antifungal agents on amylose CSPs using hexane–2-propanol–diethyl amine (400:99:1, v/v/v) as the mobile phase

	0.5 ml/min		1.0 ml/min	
	α (−)	Rs (+)	α (−)	Rs (+)
Chiralpak AS				
Econazole	1.63	5.32	1.62	2.66
Miconazole	1.56	4.69	1.54	2.89
Sulconazole	1.48	5.68	1.48	3.08
Chiralpak AD				
Econazole	1.05	1.42	1.05	0.37
Miconazole	1.06	1.26	1.06	0.32
Sulconazole	1.16	3.60	1.04	1.36
Chiralpak AR				
Econazole	1.07	0.45	1.07	0.40
Miconazole	1.05	0.32	nr	
Sulconazole			nr	

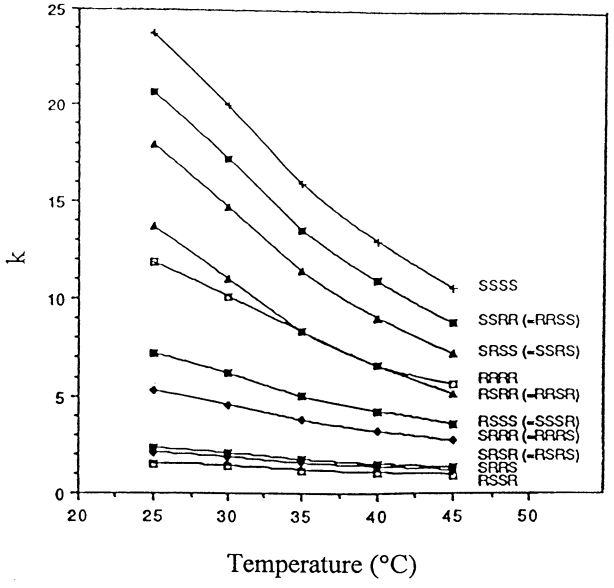


Fig. 8. Effect of temperature on resolution factor of nebivolol enantiomers on Chiralpak AD column using ethanol as mobile phase [23].

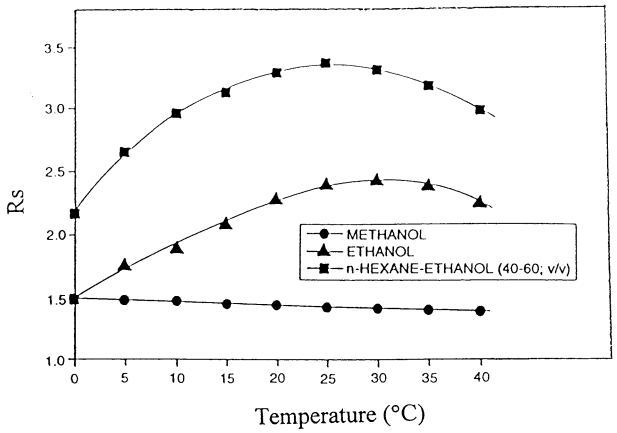


Fig. 9. Effect of temperature on resolution of benztriazole derivatives on Chiralcel OJ column using methanol, ethanol and hexane–ethanol (40:60, v/v) as the mobile phases separately and respectively [23].

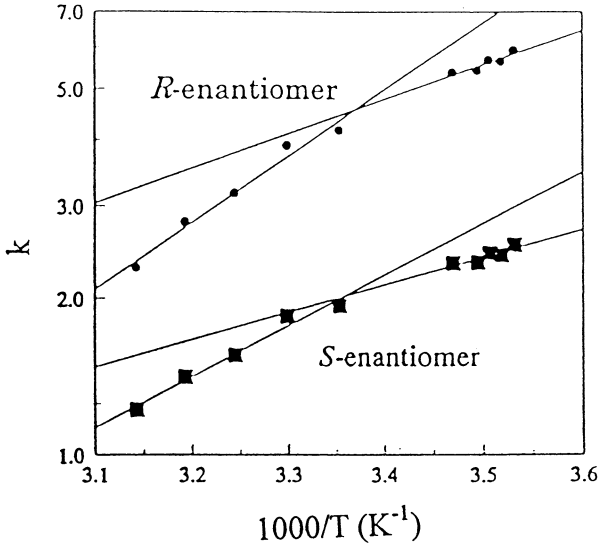


Fig. 10. Effect of temperature (van't Hoff plot) on resolution of *R,S*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-*a*]-pyrazine-4-spiro-3′-pyrrolidine)-1,2′,3,5′-tetrone on Chiralpak AD-RH column using 0.01 M acetate buffer (pH 4.7)–acetonitrile (50:50, v/v) as the mobile phase [36].

3.5. Other HPLC parameters

Besides, the optimization of HPLC factors as discussed above, the chiral resolution may be improved by adjusting the other parameters. Okamoto et al. [37]

recently studied the effect of pore size of silica gel, coating amount and coating solvent on chiral discrimination of some aromatic racemates. They concluded that CSP with a silica gel having a large pore size and small surface area showed higher chiral recognition. CSP coated with acetone as the coating solvent was found to show good chiral resolution capacity. The resolution has been improved by preparing the new derivatives of cellulose and amylose, which contain the groups capable for good capacities of bondings with racemates [38]. Recently, we have observed very interesting results by resolving methylphenidate racemates. The partial resolution of methylphenidate on Chiralcel OB has been improved to the complete resolution by using phenol or benzoic acid separately as mobile phase additives [39].

4. Optimization of HPLC conditions of enantiomeric resolution on macrocyclic glycopeptide antibiotic based CSPs

The antibiotic CSPs may be used in normal phase, reversed phase and new modified polar organic phase modes. As in case of polysaccharides based CSPs, mobile phase composition, pH of mobile phase, flow rate, temperature and other parameters are the most important HPLC conditions responsible for enantiomeric resolution on antibiotic based CSPs.

4.1. Mobile phase compositions

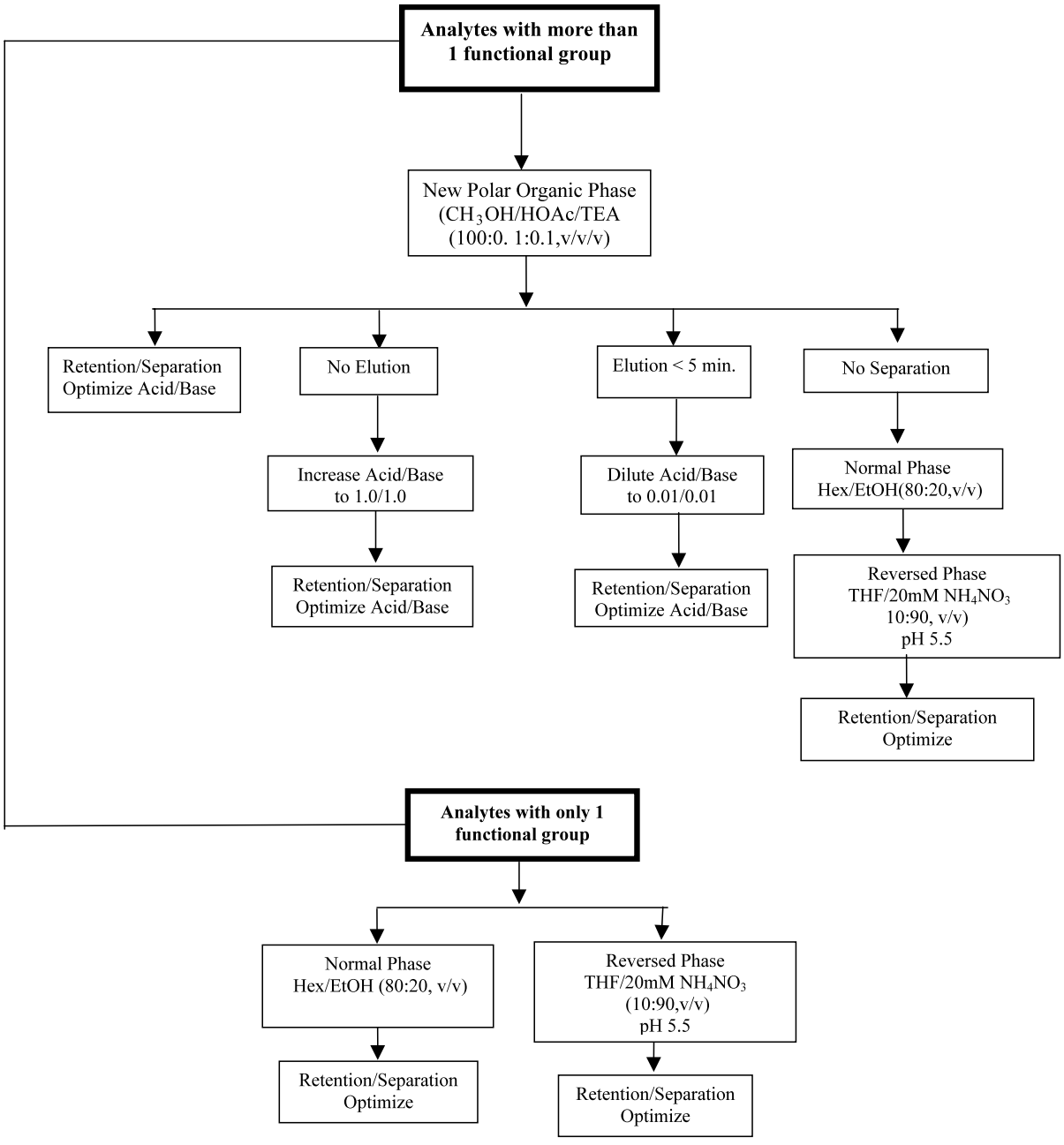
Due to the complex structure of the antibiotics, most of the antibiotics function equally well in reversed, normal and a modified polar organic phases. All the three solvent modes generally show different selectivity with different analytes. Sometimes equivalent separations were obtained in both normal and reversed phases. This ability to operate in two different solvent modes is an advantage in determining the best preparative methodology where sample solubility is a key issue. In normal phase chromatography, the typical most common solvents used are hexane, acetonitrile, ethanol, methanol etc. The optimization of chiral resolution is achieved by adding some other organic solvents such as acetic acid, THF. The protocols for the development and optimization of mobile phases on vancomycin, teicoplanin and ristocetin CSPs are shown in Schemes 3–5, respectively. The effect of the composition of mobile phases on the enantiomeric resolution of different racemates on a normal phase column is shown in Fig. 11.

In reversed phase system, mostly buffers are used as the mobile phases with a small amount of organic modifiers. The use of buffers as the mobile phases has increased the efficiency of the resolution. Ammonium nitrate, triethylammonium acetate (TEAA), sodium citrate buffers have been used very successfully. A variety of organic modifiers have been used to alter selectivity. Acetonitrile, methanol, ethanol, 2-propanol and tetrahydrofuran (THF) have shown good selectivities for various analytes. In reversed phase mode, the amount of organic modifiers is typically very low usually of the order 10–20%. The typical starting composition of mobile phase is organic modifier–buffer (pH 4.0–7.0) (10:90). Using alcohols as the organic modifiers, generally, require higher starting concentrations i.e. 20% for comparable retention when using acetonitrile or THF in starting concentration of 10%. The effect of organic solvents on the enantioselectivities also depends on the type of antibiotics. For vancomycin, low concentration of organic solvents did not significantly influence the separation while enantioresolution is improved for some compounds with ristocetin A and teicoplanin [40] even at low concentrations of organic modifiers. Fig. 12 shows the effect of the organic modifiers on the enantioselectivities of different enantiomers in reversed phase LC.

A simplified approach has proven very effective for the resolution of a broad spectrum of racemate analytes. The first consideration in this direction is the structure of the analytes. If the compound has more than one functional group capable of interacting with the stationary phase and at least one of those groups is on or near the stereogenic center, then the first mobile phase choice would be the new polar organic phase. Due to the strong polar groups present in the macrocyclic peptides, it was possible to convert the original mobile phase concept to 100% methanol with the acid/base added to effect selectivity. The key factor in obtaining complete resolution is still the ratio of acid to base. The actual concentration of acid and base only affects the retention. Therefore, starting with a 1:1 ratio some selectivity is typically observed then different ratios of 1:2 and 2:1 are applied to note the change in resolution indicating the trend. If the analyte is eluting too fast, the concentration of acid/base is reduced. Conversely, if the analyte is too well retained, the acid/base concentration is increased. The parameters for concentration are between 1 and 0.001%. Above 1% the analyte is too polar and indicates a typical reversed phase system while below 0.001% indicates a normal phase system. Both trifluoroacetic acid (TFA) and acetic acid have been used as the acid component with ammonium hydroxide as the base. For an analyte that have only one functional group or for reasons of solubility, typical normal phase solvents (hexane/ethanol) or reversed phase solvents (THF/buffer) are employed.

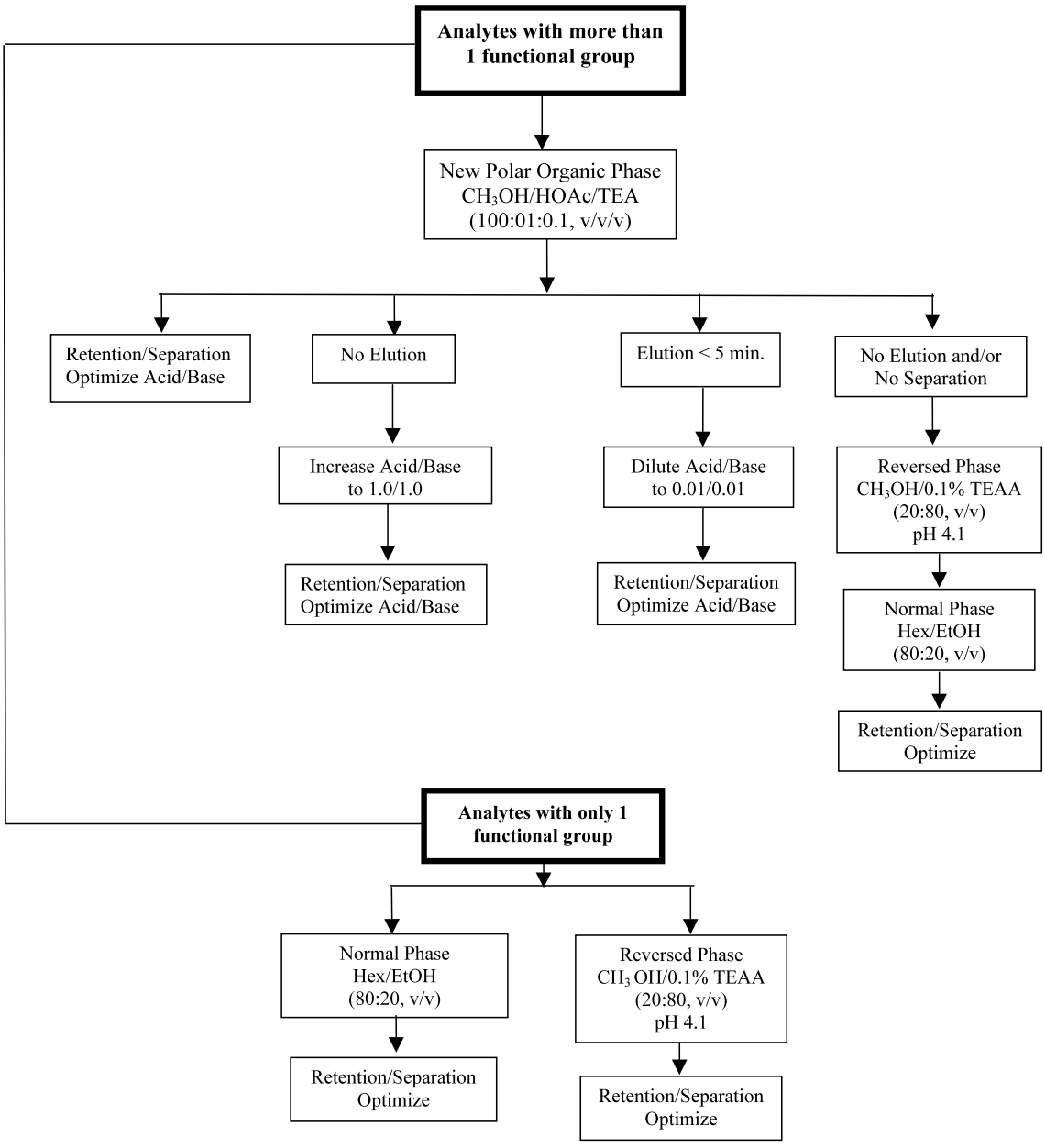
4.2. pH of mobile phase

The pH is an important controlling factor of enantiomeric resolution in normal, reversed and new organic phases. Generally, buffers are used as the mobile phases to control the pH in HPLC. The pH ranges from 4.0 to 7.0 in the reversed phase system. In general, the analytes interact more favorably at a pH where they are not ionized. Therefore, retention and selectivities of molecules that possess ionizable acidic or basic functional groups can be effected by altering the pH. A strategy may be to take advantages of a difference in pK_a values, that is, keeping the analyte of interest neutral and strongly interacting while keeping other components ionized and poorly retained. Because of the complexities of these interactions, it is necessary to observe the retention and resolution as the function of pH, usually testing at pH 4.0 and 7.0 or 0.50 pH units above and below the pK_a value. It has been observed that with the increasing values of pH the values of Rs, k and α decreases in most of the cases. Therefore, the safest and the suitable values of pH in reversed phase



Note: This is the brief outline of the procedure to follow in developing a resolution on vancomycin CSP

Scheme 3. The protocol for the development and optimization of mobile phases on vancomycin CSP.



Note: This is the brief outline of the procedure to follow in developing a resolution on teicoplanin CSP. However, the other mobile phases may be used.

Scheme 4. The protocol for the development and optimization of mobile phases on teicoplanin CSP.

systems varies from 4.0 to 7.0 [41,42]. The enantiomeric resolution by normal phase and new modified polar organic phases has been achieved below pH 7.0. The effect of the pH on Rs, k and α is given in Table 3.

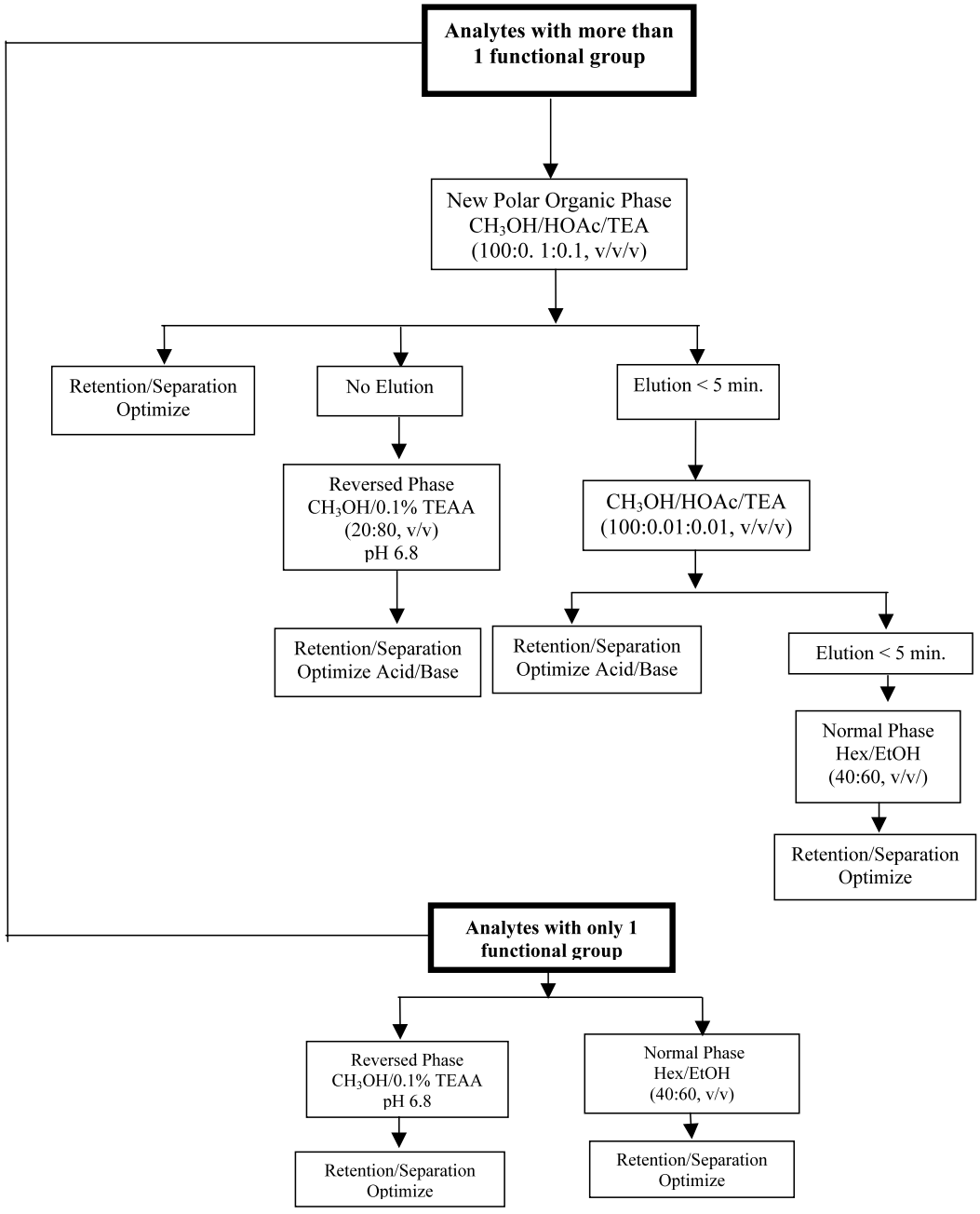
4.3. Flow rate

Armstrong et al. [40] studied the effect of flow rate on the resolution of 4-hydroxy-5-cyano-6-methoxy-3,4-dihydro-2-pyridone and α-methyl-α-phenylsuccinamide on teicoplanin column. It has been observed that flow rate does not effect the enantioselectivity (α) but does effect the separation efficiency. This is reflected by the inverse relationship between Rs and flow rate. It has also been observed that decreasing the flow rate from 2.0 to 1.0 ml/min enhanced the resolution by 20–30%. Further, decrease in the flow rate did not produce any increase in the resolution. No increase in the resolution was obtained below 0.5 ml/min flow rate in any separation mode. The loss of efficiency at higher flow rates has also been observed with most of the chiral antibiotics. The enantioselectivity, of some of the

derivatives of amino acids, on teicoplanin phase has been enhanced by simply lowering the flow rates [42]. The effect of the flow rate on the normal phase enantiomeric resolution of 3*a*,4,5,6-tetrahydrosuccinamido[3,4-*b*]acenapthen-10-one on vancomycin has been studied by Armstrong et al. [42]. They have varied the flow rates from 0.5 to 2.0 ml/min and observed that the values of α decreased from 1.31 to 1.29 while the values of Rs decreased from 1.28 to 1.11. The effect of flow rates on enantioselectivity of various racemates is given in Table 4.

4.4. Temperature

Temperature is also an important parameter for determining the resolution of enantiomers in HPLC. Armstrong et al. [42] has studied the effect of temperature on the resolution behavior of proglumide, 5-methyl-5-phenylhydantoin and *N*-carbamyl-D-phenylalanine on vancomycin column. The experiments were carried out from 0 to 45 °C. It has been observed that the values of k, α and Rs for all the three studied molecules have decreased with the increase in



Note: This is the brief outline of the procedure to follow in developing a resolution on CSP.

Scheme 5. The protocol for the development and optimization of mobile phases on ristocetin CSP.

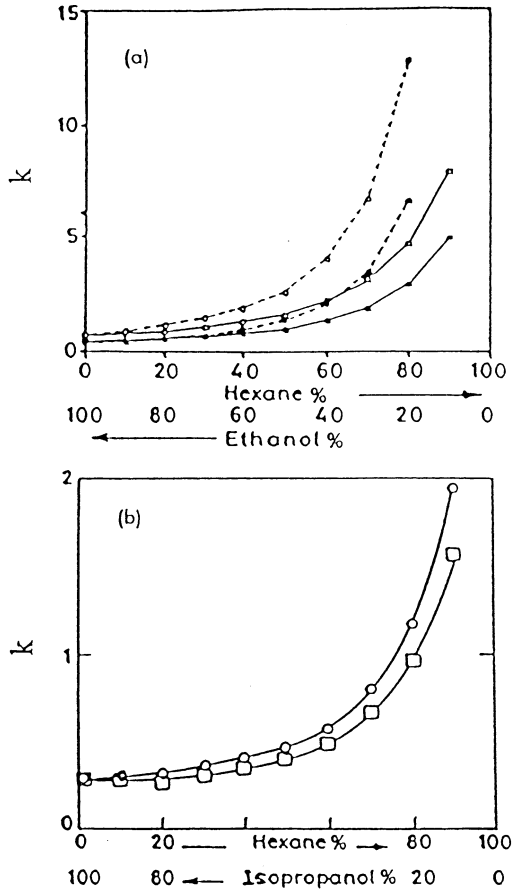


Fig. 11. The effect of mobile phase composition on the resolution of enantiomers of different racemates in normal phase mode HPLC on antibiotic CSPs. (a) First (■) and second (□) eluted enantiomers of γ-phenyl-γ-butyrolactone and first (●) and second (○) eluted enantiomers of 4-phenyl-2-methoxy-6-oxo-2,4,5,6-tetrahydropyridine-3-carbonitrile on Chirobiotic T column. (b) First (□) and second (○) eluted enantiomers of mephenytoin on Chirobiotic V column [40,42].

temperature indicating the enhancement of chiral resolution at low temperature. In another work the same workers [43] have also studied the effect of temperature on the resolution of certain amino acid derivatives on teicoplanin CSP. They further observed poor resolution at ambient temperature while the resolution increased at low and high temperature. The increase in the resolution at higher temperature may be due to the increase in efficiency of the column. It has also been observed that the change in temperature has a greater effect on the retention of solutes in normal phase in comparison to reversed phase. It may be due to the binding constant of a solute to the macrolide involves several interactive mechanisms that dramatically change with temperature. Inclusion complex formation is effectively prevented for most solutes in the temperature range of 60–80 °C. Lower temperature enhances the weaker bonding forces and the net result is that the chromatographers have an additional powerful means to control selectivity and retention. The effect of temperature in

Table 3
The effect of pH on chiral resolution of several racemates on Chirobiotic V and Chirobiotic T CSPs using methanol–1% TEAA buffer (20:80, v/v) and acetonitrile–1% TEAA buffer (10:90, v/v) as mobile phases, respectively

pH	k_1	α	Rs
5-(4-Hydroxyphenyl)-5-phenylhydantoin			
7.0	4.29	1.30	1.12
6.0	3.78	1.35	1.25
5.0	3.38	1.38	1.36
4.1	3.10	1.40	1.70
3.6	1.82	1.31	1.10
5-Methyl-5-phenylhydantoin			
7.0	0.97	2.34	2.58
6.0	0.95	2.30	2.64
5.0	0.92	2.28	2.72
4.1	0.87	2.11	2.87
3.6	0.70	1.87	2.18
Mandelic acid			
7.0	0.14	6.57	2.75
6.0	0.28	4.96	2.89
5.0	0.36	4.34	2.98
4.1	0.46	3.28	3.17
3.6	0.40	2.35	2.43

HPLC on enantioselectivity of a variety of racemates is given in Fig. 13.

4.5. Other HPLC parameters

Apart from the above parameters discussed for HPLC optimization of chiral resolution on antibiotic CSPs, some other HPLC conditions may be controlled to improve chiral resolution on these CSPs. The effect of the concentrations of antibiotics (on stationary phase) on enantioresolution varied depending on the type of racemates. The effect of the concentrations of teicoplanin has been studied on the retention (k), enantioselectivity (α), resolution (Rs) and theoretical plate number (N) for five racemates [40]. An increase in the concentration of teicoplanin resulted into an increase of α and Rs values. The most surprising fact is that the theoretical plate number (N) increases with increasing the concentration of teicoplanin. It may be due to the resistance to mass transfer resulted from analyte interaction with free silanol and/or the linkage chains (antibiotics linked with silica gel). This would tend to trap an analyte between the silica surface and the bulky chiral selector adhered to it. This is somewhat analogous to the effect of stationary phase adsorption on the efficiency of a chiral separation in micellar chromatography [44]. A more dense surface coverage of selector could prevent this deep penetration by steric means. This would tend to limit the analyte interactions to surface interaction with the chiral selector alone, thereby enhancing efficiency and possibly effecting

selectivity in some cases. It is apparent that the surface coverage and the orientation of the stationary phase are important for these separations. However, there are few reports in the literature indicating the effect of surface coverage for any CSP [40]. The effect of the concentrations of antibiotics on the retention (k), enantioselectivity (α), resolution (Rs) and theoretical plate number (N) is given in Table 5.

5. Conclusion

Polysaccharides and antibiotic based CSPs are very important for the chiral resolution of a variety of racemates. The chiral resolution can be optimized and achieved by fixing the HPLC conditions as discussed above. Normally, hexane and 2-propanol mixture is the best mobile phase for normal phase polysaccharides based CSPs. On the other hand water and acetonitrile has been used as the frequent eluent in reversed phase mode for polysaccharides based CSPs. Generally, higher pH of mobile phase on normal phase and lower pH in reversed phase modes of polysaccharides CSPs favor the chiral resolution. Flow rate of 0.5–1.0 ml/min are ideal for chiral resolution of racemic drugs on polysaccharides CSPs. The favorable working temperature with the polysaccharides CSPs is 25–30 °C. In macrocyclic antibiotics CSPs, 100% methanol for new polar organic phase, hexane, acetonitrile, ethanol, methanol for normal phase and buffers for reversed

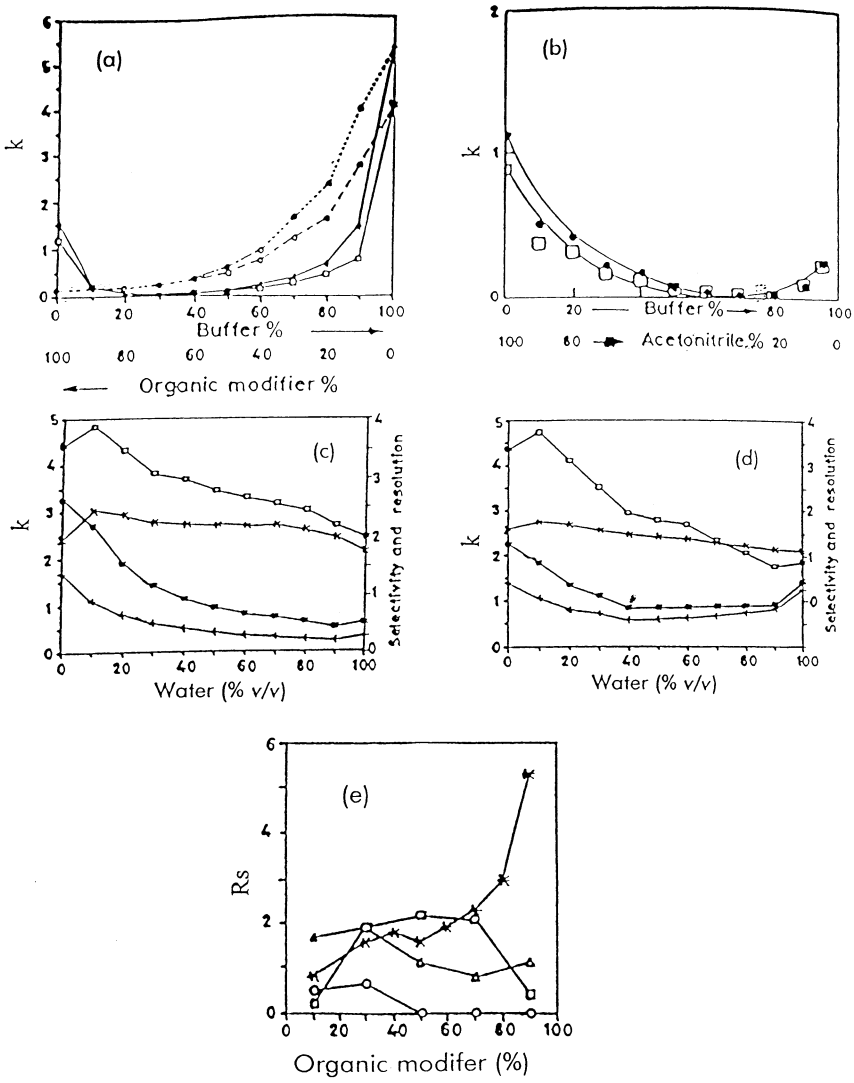


Fig. 12. The effect of mobile phase composition on the resolution of enantiomers of different racemates in reversed phase mode HPLC on antibiotic CSPs. (a) First (□,○) and second (◆,●) second enantiomers of 5-methyl-5-phenylhydantoin on Chirobiotic T column using acetonitrile–TEAA buffer (—) or methanol–TEAA buffer (----) as mobile phase. (b) First (□) and second (●) enantiomers of 5-methyl-5-phenylhydantoin on Chirobiotic V column. (c, d) First (+) and second (●) eluted enantiomers, and of resolution (□) and selectivity (x) for methionine (c) and phenylalanine (d) on Chirobiotic T column. (e) The dependence of the resolution of the enantiomers of fluoxetine (prozac) on the amount of methanol (□), 2-propanol (x), acetonitrile (○) and THF (△) as organic modifiers [40,42].

Table 4
Effect of flow rate on enantiomeric resolution on Chirobiotic V and Chirobiotic T CSPs using propanol–hexane (50:50, v/v) and ethanol–hexane (30:70, v/v) as mobile phases, respectively [40,42]

Flow rate (ml/min)	α	Rs
3*a*,4,5,6-Tetrahydrosuccinimido[3,4-*b*]acenaphthen-10-one		
0.50	1.31	1.28
0.75	1.31	1.19
1.00	1.27	1.14
1.50	1.30	1.13
2.00	1.29	1.11
4-Hexyl-5-cyano-6-methoxy-3,4-dihydro-2-pyridone		
0.50	1.4	2.4
1.0	1.4	2.3
1.5	1.4	2.0
2.0	1.4	1.6
α-Methyl-α-phenylsuccinamide		
0.5	1.3	1.9
1.0	1.3	1.8
1.5	1.3	1.6
2.0	1.3	1.4

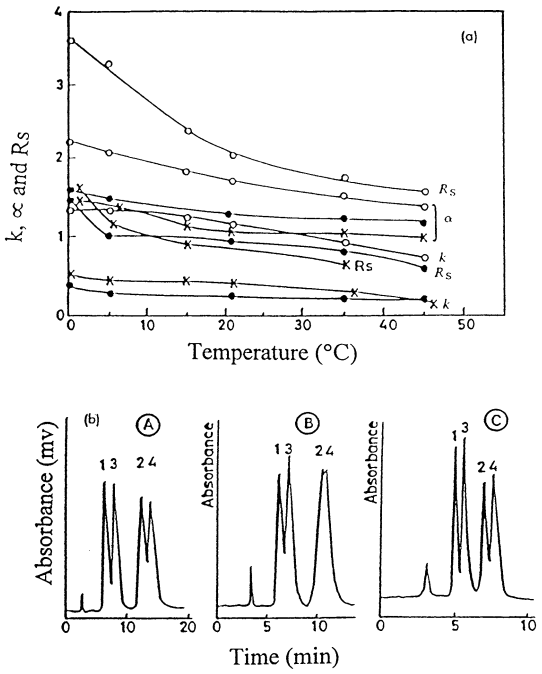


Fig. 13. The effect of temperature on enantiomeric resolution on antibiotic CSPs. (a) k, α and Rs for proglumide (○), 5-methyl-5-phenylhydantoin (●) and *N*-corbyl-DL-phenylalanine (x) on Chirobiotic V column using acetonitrile–1% TEAA buffer (10:90, v/v) as the mobile phase. (b) Separation of enantiomers of β-methyl phenylalanine on Chirobiotic T column using water–methanol (10:90, v/v) as the mobile phase with: (A) 1; (B) 20; (C) 50 °C temperatures, respectively. 1, *erythro*-L; 2, *erythro*-D; 3, *threo*-L; and 4, *threo*-D [40,43].

phase modes are used as the mobile phases. The optimization can be achieved by using acid, base or both as organic modifiers. The resolution can be controlled by adjusting the pH. The selection of pH of the mobile phase depends on the properties of the racemic drugs. The pH on antibiotic CSPs ranged from 4 to 7. The best flow rate on antibiotic CSPs are 0.5–2.0 ml/min. However, some resolution have been achieved at higher flow rate (3–4 ml/min) [41]. Again the best temperature ranged from 25 to 30 °C. In addition to these parameters, the optimization of chiral resolution on these CSPs can be achieved by varying other HPLC conditions such as particle size of CSP, pore size of column, concentration of racemic drugs and choice of suitable detector.

Acknowledgements

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Table 5
The effect of the teicoplanin concentration (x), on the column, on enantioselectivity in HPLC using acetonitrile–TEAA buffer of pH 4.1 (10:90, v/v) as mobile phase

Racemates	x	k_1	α	Rs	N (m^{-1})
	1.5	1.72	1.3	1.7	32 300
Bromacil	1.0	0.94	1.2	1.5	26 900
Dansyl methionine	1.5	3.6	1.4	2.4	40 680
	1.0	2.10	1.3	1.7	29 300
Mandelic acid	1.5	0.40	2.3	2.3	31 100
	1.0	0.37	1.6	2.0	21 500
5-Methyl-5-phenylhydantoin	1.5	0.78	1.7	2.3	34 500
	1.0	0.46	1.4	1.9	28 600
3-Phenylphthalide	1.5	2.87	1.3	2.3	25 100
	1.0	1.57	1.2	1.8	20 900

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