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Immobilisation and evaluation of a vancomycin chiral stationary phase for capillary electrochromatography

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Abstract

The macrocyclic antibiotic, vancomycin, is covalently bonded to LiChrospher® diol silica packed columns and evaluated in capillary electrochromatography (CEC) both in the reversed-phase and polar organic mode. Initially, capillaries were packed with 5 μm LiChrospher® 100 Å diol silica and evaluated in CEC with a reversed-phase biphenyl-pyrene achiral test resulting in resolution and efficiency values of ca. 2.5 and 100 000 plates meter^{-1}, respectively. Repeatability for this test (resolution and efficiency) was also examined and found to be acceptable for both run-to-run (n=5, 0.74% and 1.5%) and column-to-column (n=5, 3.4% and 9.0%), respectively. Similar results were obtained when the 10 μm LiChrospher® 1000 Å diol silica was examined with the exception of efficiency, where a reduced plate height value of four times lower was obtained compared to the 100 Å material. A simple three step in-situ vancomycin immobilisation procedure was subsequently carried out on these packed diol columns. Selectivity was obtained for thalidomide enantiomers on this vancomycin chiral stationary phase in reversed-phase CEC with resolution and efficiency values of ca. 2.5 and 80 000 plates meter^{-1}, with acceptable repeatability (n=8) 0.9% and 3.0%, respectively. Selectivity was also obtained for thalidomide enantiomers on this phase in the polar organic mode with resolution and efficiency values of ca. 2.5 and 120 000 plates meter^{-1}, with acceptable repeatability (n=7) 0.9% and 2.0%, respectively. It was possible to deduce from a chemometric design carried out for evaluating the mobile phase component effects that organic modifier ratio, MeOH/MeCN, played a significant role in controlling both resolution and efficiency. It was also possible to separate a number of basic analytes including four β-adrenergic blocking agents in the polar organic mode albeit with lower resolution and efficiency values, ca. 1.5 and 45 000 plates meter^{-1}, respectively.

Keywords: Capillary electrochromatography; Chiral stationary phases, LC; Electrochromatography; Experimental design; Diols; Vancomycin

1. Introduction

Capillary electrochromatography (CEC), a separation technique that potentially combines the separation efficiency of capillary electrophoresis (CE) with the selectivity and sample capacity of liquid chromatography (LC) has recently generated enormous interest [1–8]. This interest is predominantly generated as a result of the mobile phase transport mechanism through the chromatographic stationary phase in CEC, which is electrodriven instead of pressure driven and thus theoretically offers a number of important advantages which are often realised through increased efficiency values and improved peak resolution [9]. It is now recognised, however, that a number of significant advances have to be made before CEC can fulfil its potential and ensure a continued interest [10].

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The potential high efficiency values that can be obtained in CEC makes this an attractive technique for the separation of enantiomeric substances. Similar to the early work in chiral separation techniques, a number of chiral mobile phase additives and chiral stationary phases (CSPs) have now been successfully evaluated in CEC. Applications using cyclodextrin additives and cyclodextrin, protein, Pirkle and cellulose derivative stationary phases have all been utilised indicating high efficiencies for various analytes [5–7]. Molecularly imprinted polymer (MIP) CSPs have also been successfully applied in CEC, as additives, packed phases and as in-situ monolithic phases [11]. These MIP monolithic phases offer advantages over traditional packed columns both for chiral and achiral applications in the pharmaceutical industry but still exhibit a number of limitations including low polymer capacity and peak efficiency [12]. A more theoretical and in depth examination of the relevant aspects for enantioselectivity in CEC has also been reported [13] which may and should aid the practical approaches reported to date. More recently new CSPs for chiral CEC have been applied, a weak anion-exchange type CSP [14] and mobile phase additives [15] based on a quinine-derived chiral selector has been evaluated for the separation of N-derivatised amino acids, a vancomycin CSP for the separation of warfarin and hexobarbital in the reversed-phase mode [16], a teicoplanin for the separation of tryptophan and dinitrobenzoyl in the reversed-phase mode [17], poly-*N*-acryloyl-L-phenylalanineethylester (Chiraspher®) and cellulose tris(3,5-dimethylphenylcarbamate) CSPs for the separation of a range of compounds in three different separation modes [18] and chiral monolithic phases for reversed-phase CEC were successfully prepared for the separation of *N*-(3,5-dinitrobenzoyl)leucine diallylamide enantiomers [19]. It is also noted that, similar to achiral CEC where comparatively few applications have been reported using non-aqueous conditions [2,20–22], there are also a limited number using non-aqueous or polar organic mobile phase for chiral CEC [15,18,23–25].

In this report we demonstrate the feasibility of preparing in-situ a vancomycin CSP for CEC. Initially capillary columns are packed with LiChrospher® diol silica and evaluated in an achiral mode to ensure adequate packing and retaining frit production. The CSP is subsequently prepared using a simple three step reaction carried out by flushing the reagents for each step through the diol silica column. The in-situ approach is adopted in preference to that of packing the well characterised commercial phase or a batch synthesis primarily so that any observed enantioselectivity could be compared to earlier similar studies in LC and supercritical fluid chromatography (SFC) carried at our laboratories. The resulting vancomycin phase is evaluated in CEC for both the reversed-phase and polar organic mode with a number of neutral, basic and acidic racemic analytes. Interactions and effects of mobile phase conditions for the polar organic mode are examined using a chemometric design in this preliminary study.

2. Experimental

2.1. Chemicals

Vancomycin hydrochloride was a gift from Eli Lilly Sweden AB (Stockholm, Sweden). Acetonitrile (MeCN), methanol (MeOH), glacial acetic acid (HOAc), sodium dihydrogenphosphate monohydrate, sodium chloride (NaCl), disodium tetraborate decahydrate and all LiChrospher® diol silica phases were purchased from E. Merck AG (Darmstadt, Germany). Triethylamine (TEA), sodium cyanoborohydride and sodium periodate were obtained from Fluka Chemie AG (Buchs, Switzerland). 2-[*N*-Morpholino]ethanesulfonic acid (MES) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Acetone was purchased from Rathburn Chemicals Ltd. (Washburn, UK). Fused silica capillaries were obtained from Polymicro Technologies Inc. (Phoenix, AZ, USA). Organic solvents were of HPLC grade. All racemic analytes were obtained from Analytical Chemistry, AstraZeneca R&D Mölndal (Mölndal, Sweden). Deionised water (18.2 MΩ) used throughout the study was taken from a Maxima water purification system (Elga, High Wycombe, UK).

2.2. Instrumentation

The capillary packing pump was a Shimadzu LC-

5A (Kyoto, Japan) that was capable of operating in both constant flow and pressure mode. The micro pump used for packed capillary conditioning and immobilisation was a μLC-500 (ISCO, Gottingen, Germany). Production of retaining frits and detection windows were performed on the CE capillary burner EK 1.2 (Electro-Kinetic Technologies, Broxburn, UK). The immobilisation process was followed using a Spectra 100 spectrophotometer (Thermo Separation Products, San Jose, CA, USA) coupled to a PE Nelson 914A Interface (Perkin Elmer, San Jose, CA, USA). All CEC experiments were conducted on the Hewlett Packard ^{3D}CE system (Hewlett Packard, Waldbronn, Germany) modified to allow pressure of up to 12 bar to both the inlet and the outlet mobile phase vials. Data was collected and analysed using the HP 3D-CE ChemStation Rev. A.05.04[273] (Hewlett Packard, Waldbronn, Germany). Chemometric optimisation was carried out using the Modde 4.0 design software (Umetri AB, Umeå, Sweden) and partial least squares was used for data analysis.

2.3. Methods

Aqueous mobile phases were prepared by combining the desired volume of organic solvent to pH controlled buffer solutions and degassed by sonication for at least ten minutes. Polar organic mobile phases were prepared by combining the desired ratio of MeCN and MeOH to which trace amounts of either HOAc and/or TEA were added. Analyte stock solutions were prepared for each analyte in MeCN at a concentration of 2.5 mg/ml (unless stated otherwise) and stored at 4°C. Analytical samples for reversed-phase CEC were then prepared by a 50% dilution of this stock solution with aqueous buffer. Analytical samples for polar organic mode were prepared in a similar fashion by a 50% dilution of stock solution with MeOH (unless stated otherwise).

2.4. Diol silica column preparation

Capillaries (75 μm) were packed with LiChrospher® diol silica in acetone (80 mg/ml) using the slurry packing technique described earlier [26]. The 5 μm LiChrospher® 100 Å and the 10 μm LiChrospher® 1000 Å were each packed in order to investigate the effects of particle size and pore dimension. The capillary outlet was fitted with a 0.5 μm porous PEEK end-fitting (Upchurch Scientific, Oak Harbor, WA, USA) and the inlet connected to a home made stainless steel packing reservoir. This slurry contained in the reservoir was magnetically stirred continuously to avoid sedimentation and the entire apparatus was totally immersed in an ultrasonic bath (Medelco, Hägersten, Sweden) to ensure adequate packing. The packing solvent, acetone, was initially delivered at 0.3 ml/min until 490 bar was reached after which, constant pressure was applied for two hours. The pressure across the column was then allowed to slowly bleed off depending on the phase packed. Prior to retaining frit production the capillary was washed with either sodium chloride (10 m*M*), disodium tetraborate (5 m*M*) or water for 45 minutes at 450 bar. The inlet frit is prepared first under 450 bar at approximately 5 cm from the capillary end attached to the PEEK end-fitting by applying high temperature (ca. 400°C) for 20 s using the capillary burner [27,28]. The PEEK end-fitting was subsequently removed to allow packing material prior to the inlet frit to be flushed to waste. The outlet frit is then prepared under pressure at the desired column length from the inlet frit and the pressure allowed to bleed off. The column is then reversed to flush away the stationary phase that is not trapped between the two retaining frits, a detection window prepared and conditioned with the desired mobile phase for one hour prior to use.

2.5. Evaluation of diol silica columns

Diol silica CEC columns were equilibrated under an applied electric field for one hour, or until a stable current and baseline were achieved. It was considered imperative that, prior to any immobilisation of vancomycin selector, the diol silica columns should be evaluated in achiral CEC and biphenyl and pyrene, both at a concentration of 0.5 mg/ml, were considered to be suitable model analytes. Linear velocity and van Deemter curves were plotted together with repeatability, resolution and retention time data (n=5–9) were used to verify selectivity and efficiency for each diol silica column packed.

2.6. Vancomycin immobilisation

The immobilisation of the vancomycin chiral selector to the diol silica was monitored at 280 nm as described for microbore liquid chromatography (μ-LC) [29]. The oxidation of the secondary hydroxyl groups of the diol to the aldehyde was carried out using a flush of 70 m*M* solution of sodium periodate in water/methanol (4:1, v/v) for two hours followed by a water flush. Immobilisation was subsequently carried out by reductive amination of the resulting aldehyde silica with a solution of 3.5 m*M* vancomycin hydrochloride and 16 m*M* sodium cyanoborohydride in 50 m*M* NaH_2PO_4 at pH 7. Remaining aldehyde moieties were reduced back to hydroxyl groups by flushing a solution of 10 m*M* sodium cyanoborohydride in 50 m*M* NaH_2PO_4 at pH 3 for two hours and later mobile phase for equilibration one hour prior to installation to the CEC instrument.

2.7. Chiral stationary phase evaluation

The vancomycin CSP was evaluated in both the reversed-phase and polar organic mode using racemic thalidomide as test analyte. Similar to the diol silica column evaluation, linear velocity and van Deemter curves were plotted to verify selectivity and efficiency on each column immobilised. Repeatability data ($n=6–8$) were also obtained. Subsequent to this evaluation a number of racemic analytes were screened in both phases and a chemometric study carried out to optimise mobile phase conditions for thalidomide in the polar organic mode.

3. Results and discussion

3.1. Diol silica column preparation

Initially, the packing of the slurry was carried out as quickly as possible to minimise the risk of slurry sedimentation but was not possible. Magnetic stirring of the slurry coupled with the lowering the reservoir and capillaries into an ultrasonic bath during the packing procedure resolved the problem and may also have prevented clogging of the silica material at the capillary inlet which can occur as described earlier [27,30]. The production of retaining frits was carried out by threading the capillary through a resistance coil and applying high temperatures [27,28]. This technique requires the stationary phase to have a high sodium content (ca. 1500 ppm) so that porous plugs of sodium silicate may be produced at sufficiently high temperatures [7]. It was not possible to prepare retaining frits on the 5 μm LiChrospher® 100 Å diol silica when the column was flushed with water prior to sintering. This was overcome by applying a wash of sodium chloride (10 m*M*) or disodium tetraborate (5 m*M*). When the concentration of either of these solutions was too high the retaining frits were often blackened and susceptible to breakage at high pressures. No difference was observed in performance between a wash of sodium chloride or disodium tetraborate at the same sodium concentration. Surprisingly, retaining frits were easily produced for the 10 μm LiChrospher® 1000 Å diol silica after a water flush, which may indicate a different silica manufacturing process for this material compared to the 100 Å phase which resulted in a higher sodium content.

3.2. Evaluation of diol silica columns

The diol silica column evaluation was intended to evaluate the packing procedure and retaining frit production before the time consuming process of immobilisation was initiated. Additionally, this was important since the evaluation of diol packed capillaries has not been reported in CEC. It was thought that a basic understanding of the chromatographic and flow properties of the diol phase was required before the vancomycin molecule was attached since the antibiotic contains ionisable groups and the final EOF on the chiral phase may not only be generated from the silica particles but additionally from vancomycin molecule.

Two LiChrospher® diol silica materials were packed, each characterised by different particle and pore dimension, a 5 μm 100 Å and a 10 μm 1000 Å. When optimising the separation conditions for the biphenyl-pyrene test system with the 5 μm 100 Å phase, MeCN content in MES buffer was evaluated. It was shown that efficiency increased while resolution and migration time decreased for higher organic modifier contents. This is similar to reports

in CEC with ODS phases [31]. TRIS and disodium hydrogen phosphate were also examined at a concentration of 50 m*M*. The former has a low buffering capacity at pH values below 7.5 but was shown to adversely effect the silica. The phosphate resulted in higher current values and was thus not chosen since this may have contributed to excess Joule heating and/or bubble formation. The pH of the MES buffer was examined and although migration time decreased at higher values resolution was unaffected. A test system that combined optimum efficiency, resolution, migration time and stability was obtained for the 5 μm LiChrospher® 100 Å phase with MeCN/50 m*M* MES, pH 6.5 (95:5, v/v), shown in Fig. 1.

A plot of linear velocity with applied field and a van Deemter curve were plotted for biphenyl on this 5 μm 100 Å material, shown in Fig. 2, where a reduced plate height value of ca. 2 (ca. 100 000 plates meter^{-1}) was shown at a linear velocity of 0.75 mm/s. The repeatability of this separation was examined resulting in RSD values for migration time, resolution, area and efficiency (n=5) of 0.2%, 0.74%, 8% and 1.5%, respectively. Column-to-column repeatability (n=5) values for resolution and efficiency were found to be 3.4% and 9%, respectively.

The effect of particle size and pore dimension was subsequently evaluated and shown to have a greater effect on efficiency than on selectivity. It was necessary, however, to re-optimise mobile phase conditions for the 1000 Å diol material, since the test analytes were eluted from the column much quicker than from the 100 Å phase under similar conditions. Similar to the 100 Å material, optimum efficiency and resolution for the 1000 Å phase was observed at a linear velocity of ca. 0.75 mm/s (ca. 190 000 plates meter^{-1}) shown in Fig. 2. The expected higher flow-rates observed with the 1000 Å phase may result from reduced mass transfer effects with the higher pore material which may account for the relatively flat van Deemter profile [32]. This profile is in contrast to that obtained for the 100 Å, which shows a rapid increase in the reduced plate height value at higher linear velocities. Additionally, higher efficiencies with reduced plate height values of 4 times lower are achieved with the 10 μm 1000 Å

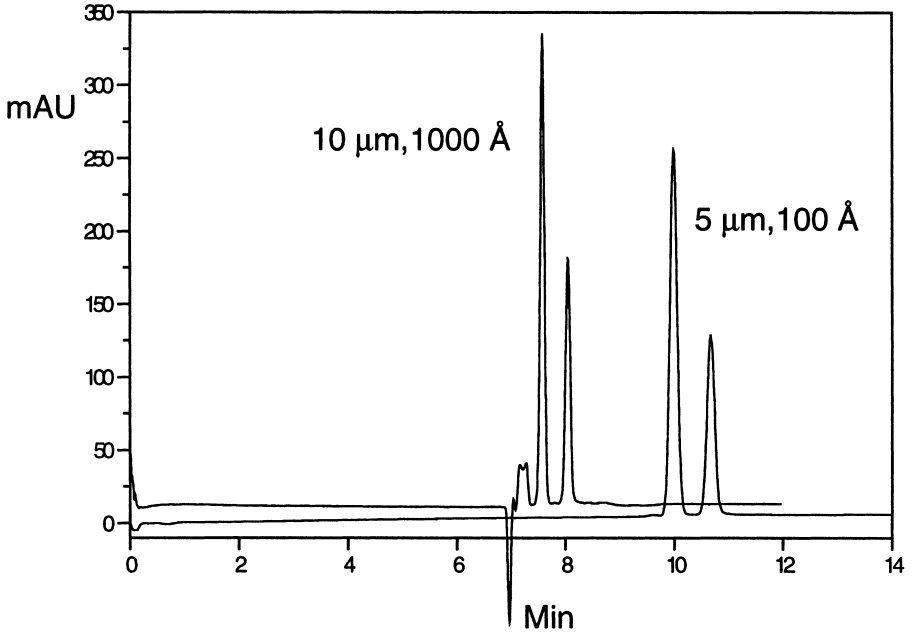

Fig. 1. CEC separation of biphenyl and pyrene on the 5 μm LiChrospher® 100 Å diol phase: MeCN/50 m*M* MES at pH 6.5 (95:5, v/v), 350 mm×75 μm I.D. (L_d 260 mm), 20 kV, 3 s injection at 5 kV, 15°C, 10 bar and detection at 200 nm and on the 10 μm LiChrospher® 1000 Å: MeCN/50 m*M* MES at pH 6.5 (40:60, v/v), 350 mm×75 μm I.D. (L_d 260 mm), 15 kV, 3 s injection at 5 kV, 15°C, 10 bar and detection at 200 nm.

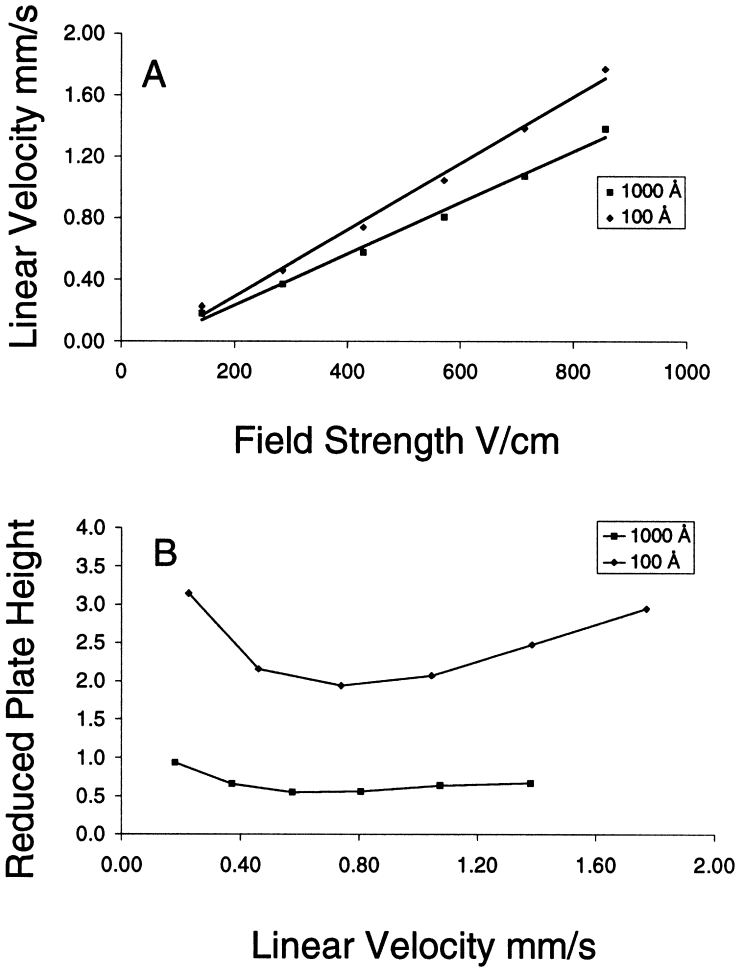

Fig. 2. Plot of (a) linear velocity with applied field and (b) van Deemter curves for biphenyl on the 5 μm LiChrospher® 100 Å and the 10 μm LiChrospher® 1000 Å diol silica. Conditions as described in Fig. 1.

material (Fig. 2b). It may be possible that in addition to mass transfer effects, the increased efficiency with the larger pore material may be a result of less double layer overlap. It has been proposed that phases having particle diameters of 0.4 μm can be applied before EOF velocity is affected [3]. This may be different, however, if porous particles are applied where the EOF generation may also occur within the pores, in which the case pore dimension may significantly contribute to observed efficiency [7]. Li and Remcho concluded from their examination of pore dimension on electrochromatographic efficiency that higher pore size material precluded excessive electrochemical double layer overlap resulting in higher efficiencies [32].

The repeatability of the biphenyl-pyrene test separation on the 10 μm LiChrospher® 1000 Å diol column was examined and RSD values for migration time, resolution, area and efficiency ($n=8$) were 0.2%, 2.9%, 6% and 4%, respectively. RSD values could not be evaluated for column-to-column repeatability since only two 1000 Å columns were packed, however, these were shown to offer repeatable chromatographic results.

Two Nucleosil® diol silica materials, 7 μm 300 Å and 7 μm 500 Å, were also packed to capillary

columns and investigated in the CEC mode. The 300 Å material packed well in a 75 μm capillary, however, it was not possible to generate an EOF. The 500 Å material possibly gave an EOF, but the current never stabilised. It is difficult to understand the poor performance of these Nucleosil® diol materials in CEC. It is believed that less free silanol moieties are available on this phase due higher diol coverage or even some endcapping which would have to be verified by the supplier. Additionally, it was noted that during the early μ-LC studies to immobilise vancomycin that it was not possible to obtain repeatable results for efficiency when these Nucleosil® diol silica materials were examined [29].

3.3. Vancomycin immobilisation

In order to immobilise the vancomycin chiral selector to the diol moieties a technique previously described has been adapted [29,33]. The first immobilisation step, the oxidation of the diol hydroxyls to aldehyde groups has been previously studied in solution with diol silica slurried in the oxidising medium. Comparison of diffuse reflectance infrared (IR) spectra of washed and dried packing material withdrawn from the reaction at different times (30 s–48 h) showed no difference in peak height of the carbonyl band [34]. This indicates that the reaction is very fast and normally completed within a minute. The UV trace of the second immobilisation step, attachment of the chiral selector to the aldehyde functions, was followed at 280 nm and shown in Fig. 3. The selector is dissolved in phosphate buffer (pH 7) together with sodium cyanoborohydride [35]. The reaction proceeds in two steps, the first being a nucleophilic attack of the amino nitrogen to form an imminium intermediate with the aldehyde. This is the slow and rate determining step. The next step where the imminium intermediate is reduced with sodium cyanoborohydride to a new amine is faster (minutes). The overall reaction time is thus complete in a few hours. The UV absorbance trace initially declines Fig. 3(a–b) due to the water flush indicating that it takes approximately 50 min for the vancomycin reaction solution to reach the detector. For a three hour period the UV trace increases slowly before it comes to a constant value, Fig. 3(c). It is thought that the vancomycin is reacting with the aldehyde functionalities between point (b) and (c) and terminates after approximately three hours. Twenty hours after the vancomycin solution was injected, water is flushed through the system (I) which is followed by the last immobilisation step the reduction of any remaining aldehyde moieties back to hydroxyl groups. The aldehyde is relatively inert towards cyanoborohydride unless the pH is low [35],

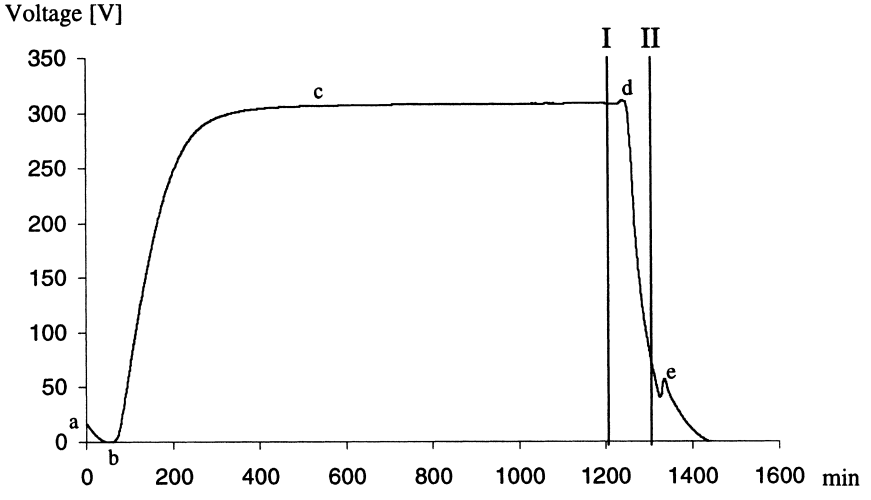

Fig. 3. 280 nm UV trace of the in-situ immobilisation of vancomycin to a 5 μm LiChrospher® 100 Å diol silica packed column. Reaction solution flushed through the column at constant pressure of 300 bar.

so phosphate buffer at pH 3 is adopted and allowed to flush through the column (II). A small peak appears shortly after this flush, Fig. 3(e), which may indicate some UV absorbance of cyanoborohydride at 280 nm.

In an earlier study, the commercial Chirobiotic V CSP was investigated by Raman spectroscopy and compared to the vancomycin hydrochloride prepared as described above. The two showed similarities and differences compared with vancomycin hydrochloride and vancomycin immobilised on 100 Å aldehyde functionalised silica. The main differences observed are likely to result from another silica material or a different linker in that immobilisation procedure The preparation of Chirobiotic V as described by Armstrong's group indicates that there may be an average of three spacers linked to each vancomycin molecule possibly at twelve different positions [34].

3.4. Chiral stationary phase evaluation

When vancomycin had been immobilised to the first 5 μm LiChrospher® 100Å diol column, it was considered imperative to find a test system so that subsequent columns could be evaluated. Thalidomide was selected as the racemic analyte, since these phases were extremely selective for this analyte in both μ-LC [29,36] and packed capillary SFC [37]. Based on the reversed-phase conditions for this phase in μ-LC, MeCN with a buffer system of triethylamine acetate (TEAA) was examined. Buffer concentration, pH and MeCN concentration were optimised resulting in an adequate reversed-phase chiral test, shown in Fig. 4. Baseline separation of thalidomide enantiomers was achieved with reasonable migration times and satisfactory efficiency values for a chromatographic chiral separation (ca. 80 000 plates meter^{-1}). Repeatability for this chiral separation was examined resulting in RSD values (n=8) for migration time, resolution, area and efficiency of 1%, 0.9%, 4.0% and 3.0%, respectively. Repeatability values for this separation from column to column (n=3) for resolution and efficiency were 2.5% and 28%. Compared to the present results, efficiency values for the first eluted enantiomer of thalidomide were much lower in reversed-phase μ-LC and SFC (ca. 8000 and 22 000 plates meter^{-1},

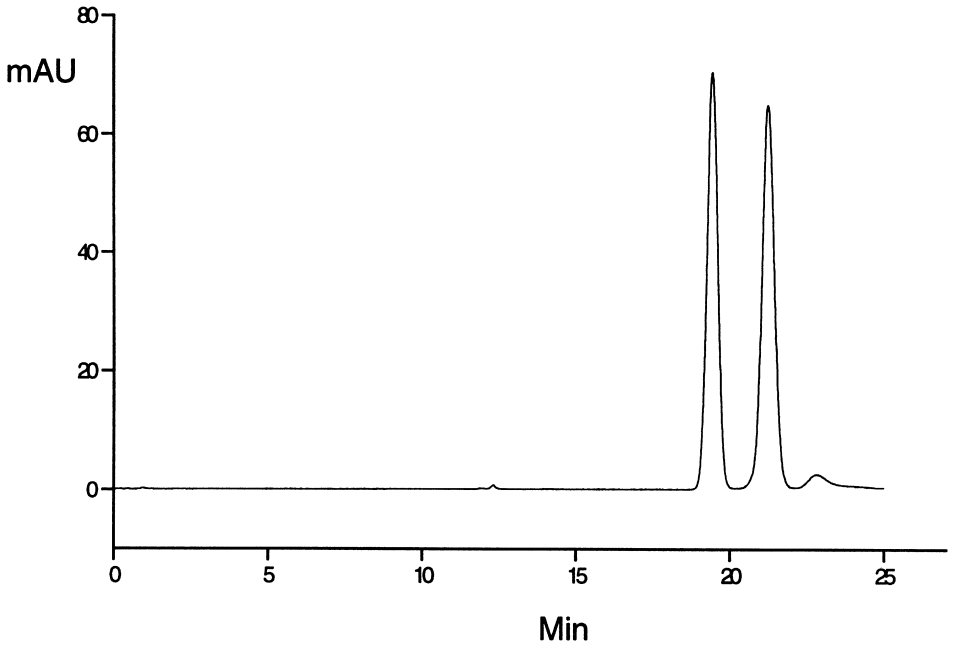

Fig. 4. Chiral CEC separation of thalidomide enantiomers on a 5 μm vancomycin 100 Å CSP. MeCN/0.1% TEAA at pH 6.5 (30:70, v/v), 353 mm×75 μm I.D. (L_d 261 mm), 15 kV, 5 s injection at 10 kV, 15°C, 10 bar pressurisation and detection at 220 nm.

respectively). These results (Fig. 4) also compare favorably with those of a recent short communication reporting the chiral separation of hexobarbital (ca. 37 000 plates meter^{-1}) in CEC using a vancomycin CSP albeit using a different immobilisation procedure [16].

Once the vancomycin CSP was evaluated in the reversed-phase mode, a number of racemic compounds were examined including, binaphthol, bupivacaine, dichloroprop, ibuprofen, ketoprofen, metoprolol, and warfarin. The results of this study were surprisingly disappointing since any small change in mobile phase conditions seemed to have a deleterious effect on the stability of the CSP and re-equilibration was extremely difficult. As a direct result of the equilibration problems, of the racemic compounds examined, only thalidomide was successfully separated. This was very surprising since this vancomycin CSP was shown to be selective for many racemic analytes in μ-LC and SFC [36–38]. During these problems using the reversed-phase mode, no column degradation was observed since each column examined was repeatedly investigated using the thalidomide test system but resolution and migration times were consistent with the original results. It was possible, however, to determine the effects of mobile phase conditions for the thalidomide separation although for a narrow window.

To examine the effect of organic modifier for the separation of thalidomide, both MeOH and MeCN were examined. No advantages of using MeOH over MeCN were observed throughout the study, with improved resolution, migration times and efficiency obtained for the latter. It was found that an efficiency and separation maximum was reached at 70% aqueous buffer which dropped off at higher and lower values. This phenomena indicating that optimum separation may be obtained at approximately 70–80% buffer in organic modifier is analogous to LC results obtained in other studies for vancomycin [39], teicoplanin [40], ristocetin A, [41] and avoparcin [42]. As expected higher pH values resulted in a greater EOF but this parameter will also determine the ionic charge of both analyte and chiral selector and thus the potential for additional or reduced enantioselectivity. This would indicate that a chiral separation for some analytes would only be possible in a very narrow pH region. During this study, however, it was changes in pH from 6.5 that contributed most significantly to the instability of the column in the reversed-phase mode. It may thus be concluded that changes in the ionic nature of the CSP were predominantly responsible for the observed instability.

Since the efficiency values for the 10 μm 1000 Å diol material were found to be superior than those for the smaller pore size material during the diol column evaluation steps, it was decided to immobilise vancomycin to this material. It was thought that the observed efficiency increase for the 1000 Å material may be transferred to higher efficiency values for chiral separations than those obtained with the 100 Å phase. A limited study of this material was carried out, but neither efficiency nor selectivity was improved with this phase over the 5 μm 100 Å vancomycin CSP. Similar to the 5 μm 100 Å CSP no chiral separation other than thalidomide was achieved in the reversed-phase mode.

3.5. Polar organic mode

There have been relatively few reports in the literature concerning the application of non-aqueous mobile phases in CEC for chiral separations [15,18,23–25] and to our knowledge no report to date specifically using the polar organic mobile phase. Polar organic mode was investigated in this study, however, since improved results with this CSP in μ-LC were obtained by Svensson et al. [38]. The basic, neutral and acidic compounds examined, conditions applied and results obtained are shown in Table 1. It was clear from these results that the 5 μm 100 Å vancomycin CSP was shown to be considerably more enantioselective in this mode than in the reversed-phase mode. It was also clear, from the data in Table 1 that, with the exception of racemic thalidomide, all compounds that had been separated contained basic functionalities. Fig. 5 shows the chromatograms for the four β-adrenergic blocking agents, alprenolol, atenolol, metoprolol and practolol. It was not possible to draw any firm conclusions or quantify the degree of resolution or efficiency from these preliminary data on the basis of structural differences. It can be noted, however, that a problem associated with CEC in general, the adsorption of

Table 1
Racemic compounds examined in the polar organic mode in CEC with the in-situ immobilised Vancomycin CSP

Compound	Mobile phase [a]	t_{m1}	t_{m2}	N_1 [b]	N_2 [b]	R_s
Alprenolol	80/20/0.1/0.2	8.3	8.9	50 000	46 000	1.89
Atenolol	20/80/0.1/0.2	13.3	14.3	41 000	46 000	1.93
Benzoin	50/50/0.3/0.2	7.6	–	91 000	–	0
Binaphthol	70/30/0.6/0.4	13.2	–	113 000	–	0
Bupivacaine	20/80/0.3/0.1	6.8	7.4	64 000	29 000	2.17
Dichloroprop	50/50/0.2/0.1	No peaks detected				
Ephedrine	20/80/0.3/0.1	13.0	13.3	4000	4000	0.24
Ibuprofen	50/50/0.2/0.1	No peaks detected				
Isoprenaline	50/50/0.2/0.2	7.3	7.8	8000	12 000	0.67
Ketamine	20/80/0.3/0.1	4.8	5.9	40 000	42 000	1.23
Ketoprofen	50/50/0.1/0.2	10.6	–	35 000	–	0
Metaqualone	50/50/0.3/0.2	7.6	–	72 000	–	0
Methadone	20/80/0.3/0.1	5.1	–	45 000	–	0
Methoxyfenandrin	50/50/0.1/0.3	5.4	–	9000	–	0
Metoprolol	80/20/0.1/0.2	7.1	7.9	76 000	74 000	1.85
Phenylamine	20/80/0.1/0.2	10.9	11.4	55 000	42 000	1.21
Practolol	80/20/0.1/0.2	12.6	13.3	43 000	34 000	1.35
Thalidomide	80/20/0.2/0.2	12.3	13.1	115 000	110 000	2.52
Ornidazole	50/50/0.2/0.2	6.9	–	98 000	–	0
Warfarin	50/50/0.2/0.1	14.8	–	27 000	–	0

[a] Mobile phase expressed as MeOH/MeCN/HOAc/TEA, (v,v,v,v).
[b] Efficiency is expressed as plates meter^{-1}.

basic analytes to the anionic silica particles resulting in peak tailing [43,44], is evident in these chiral examples despite the addition of trace quantities of competing base (TEA) and the presence of a chiral selector. Similar resolution values were also obtained for these analytes when examined in μ-LC under polar organic conditions, although peak efficiencies were considerably lower (ca. 5000 plates meter^{-1} for metoprolol) [36] compared to those obtained in these studies in CEC (ca. 76 000 plates meter^{-1} for metoprolol).

It is also shown in Table 1, that enantioselectivity was not achieved, with the exception of thalidomide, for either acidic or neutral analytes. Similar to the poor results obtained in the reversed-phase mode, it is clear that the ionic state of the analyte plays a crucial role for obtaining enantioselectivity. It is clear that this in-situ immobilised vancomycin CSP is strongly affected when a field is applied since acceptable results, for acidic, basic and neutral analytes, are obtained in reversed-phase μ-LC [36] and for acids in the polar organic mode [39].

Although it was shown that this CSP was extremely selective for thalidomide in reversed-phase, normal-phase and polar organic mode μ-LC in addition to SFC [34,36–38], it is still difficult to explain the anomalous results obtained in CEC for this molecule compared to others. The chiral separation of thalidomide in the polar organic mode is shown in Fig. 6, indicating the only acid successfully separated (R_s=2.5) in these studies coupled with exceptional efficiency values (ca. 120 000 plates meter^{-1}) for a chromatographic chiral separation. The retention time of these enantiomers are quite low indicating that they may be eluting with the EOF. This is difficult to state unequivocally since no unretained EOF marker was added to any analyte, however, retention times are longer than those of benzoin or binaphthol which are both neutral. A separate study would have to be carried out to investigate and choose an appropriate unretained EOF marker.

It was decided to investigate the role of mobile phase factors, organic modifier, competing base and acid concentrations, for the separation of thalidomide enantiomers by the use of a statistical experimental design in favor of the more traditional univariate approach. An advantage of this approach would be in addition to obtaining optimal operating conditions,

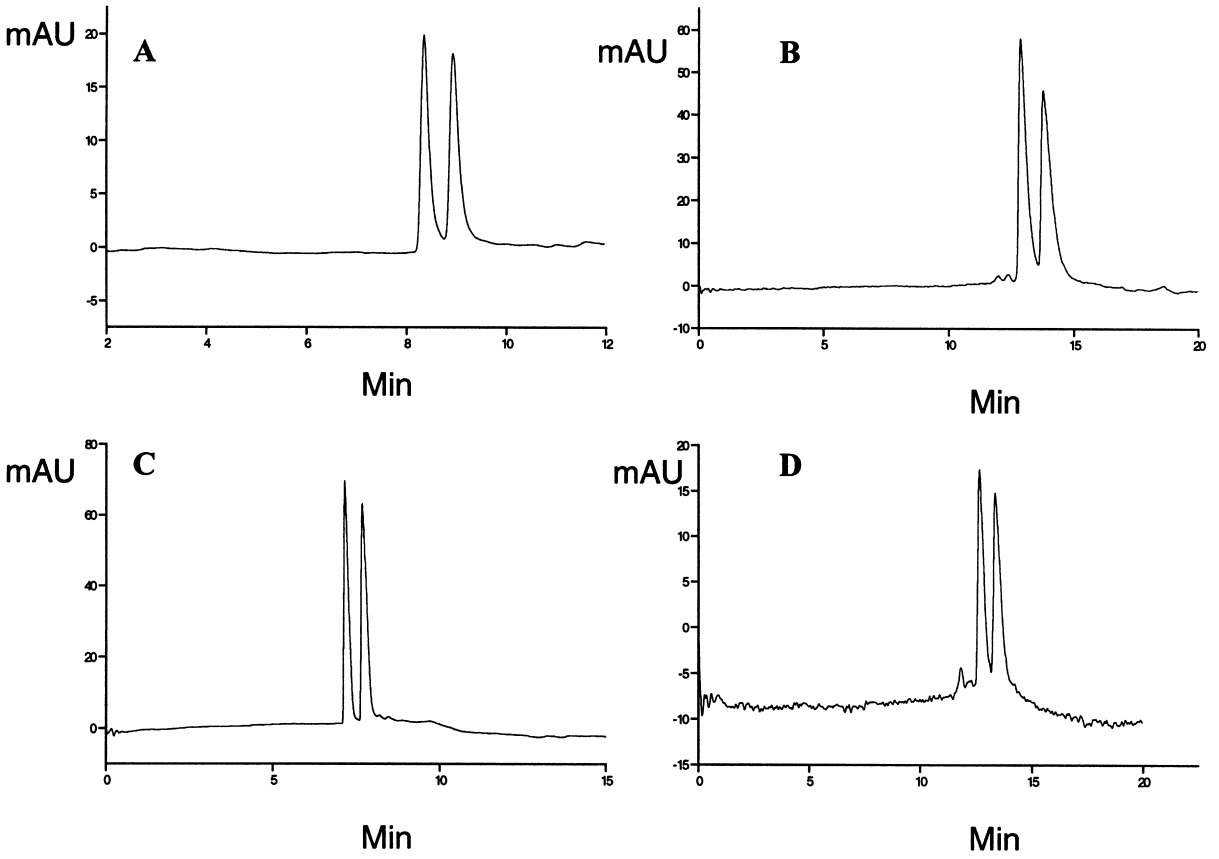

Fig. 5. Chiral separation of the four β-adrenergic blockers (a) alprenolol, (b) atenolol, (c) metoprolol and (d) practolol using the polar organic mode mobile phase. MeOH/MeCN/HOAc/TEA, (a) 80:20:0.2:0.2, v,v,v,v (b) and (d) 80:20:0.1:0.2, v,v,v,v and (c) 50:50:0.1:0.3, v,v,v,v. 355 mm×75 μm I.D. (L_d 265 mm), 10 kV, 3 s injection at 5 kV, 15°C, 10 bar and detection at 200 nm.

an estimate of the factor interactions would also be attained. The mobile phase consisted of a mixture of two organic solvents, MeOH and MeCN, and an acid base pair, HOAc and TEA. Since the percentage MeCN could be calculated as 100 − %MeOH, the three factors chosen were: MeOH content, HOAc and TEA. Acid/base pairs other than HOAc/TEA were not examined in this preliminary study. At first the intention was to use four CEC responses, migration time, resolution, selectivity and efficiency but migration time and selectivity were excluded from the final models since the former was low enough for every run and it was found that the latter followed a similar pattern to that of resolution. A run sequence was generated by the statistical program (Modde version 4.0) which is shown in Table 2 together with response results at each factor point. A summary of fit plot for efficiency (N_c) and resolution (R_s) was calculated by the software to verify each model. Resolution was found to give the best fit with an R^2 (coefficient of determination) value of 0.96, and a Q^2 (cross-validated correlation coefficient) value of 0.76, however, the values for efficiency are still acceptable (R^2=0.86 and Q^2=0.61). From these experimental design data it was possible to generate response surface plots, shown in Fig. 7 for the two responses, in order to deduce if any factor interaction effects existed. It is clear from both sets, Fig. 7a and 7b, for resolution and efficiency respectively, that for the separation of thalidomide enantiomers, the predominant factor is organic modifier content. The responses for resolution (Fig. 7a) indicate an increase

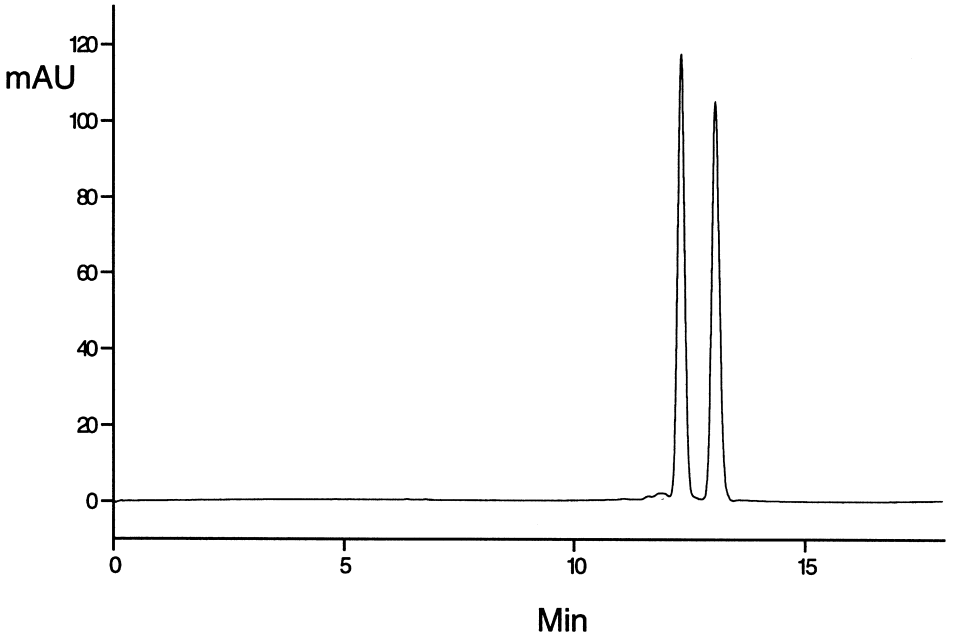

Fig. 6. CEC separation of thalidomide enantiomers on a vancomycin CSP in the polar organic mode. MeOH/MeCN/HOAc/TEA, (80:20:0.2:0.2, v,v,v,v), 355 mm×75 μm I.D. (L_d 265 mm), 20 kV, 3 s injection at 10 kV, 15°C, 10 bar and detection at 220 nm.

Table 2
Experimental variables and responses obtained for the chemometric evaluation of the CEC chiral separation of thalidomide on the vancomycin CSP in the polar organic mode. Modifier conditions are calculated as MeOH/MeCN (X: 100−X, v,v) and HOAc/TEA concentrations are also expressed as volumes

Exp. No.	Run order	Methanol	HOAc	TEA	Efficiency[a] (N_c)	Resolution (R_s)
1	14	20	0.1	0.1	12	1.34
2	3	80	0.1	0.1	16	1.93
3	5	40	0.3	0.1	19	1.15
4	19	70	0.5	0.3	21	1.71
5	1	20	0.6	0.1	12	1.18
6	8	80	0.6	0.1	16	1.71
7	20	60	0.1	0.2	26	1.68
8	10	80	0.3	0.2	19	2.09
9	6	30	0.6	0.2	16	1.14
10	9	20	0.6	0.3	14	1.37
11	15	60	0.4	0.3	21	1.45
12	11	20	0.1	0.4	13	1.57
13	13	80	0.1	0.4	16	2.18
14	18	60	0.2	0.4	20	1.58
15	7	40	0.5	0.4	21	1.32
16	12	20	0.6	0.4	14	1.33
17	4	80	0.6	0.4	21	2.11
18	17	50	0.3	0.2	27	1.44
19	2	50	0.3	0.2	25	1.32
20	16	50	0.3	0.2	23	1.26

[a] Efficiency is expressed as plates column^{-1}×10^3.

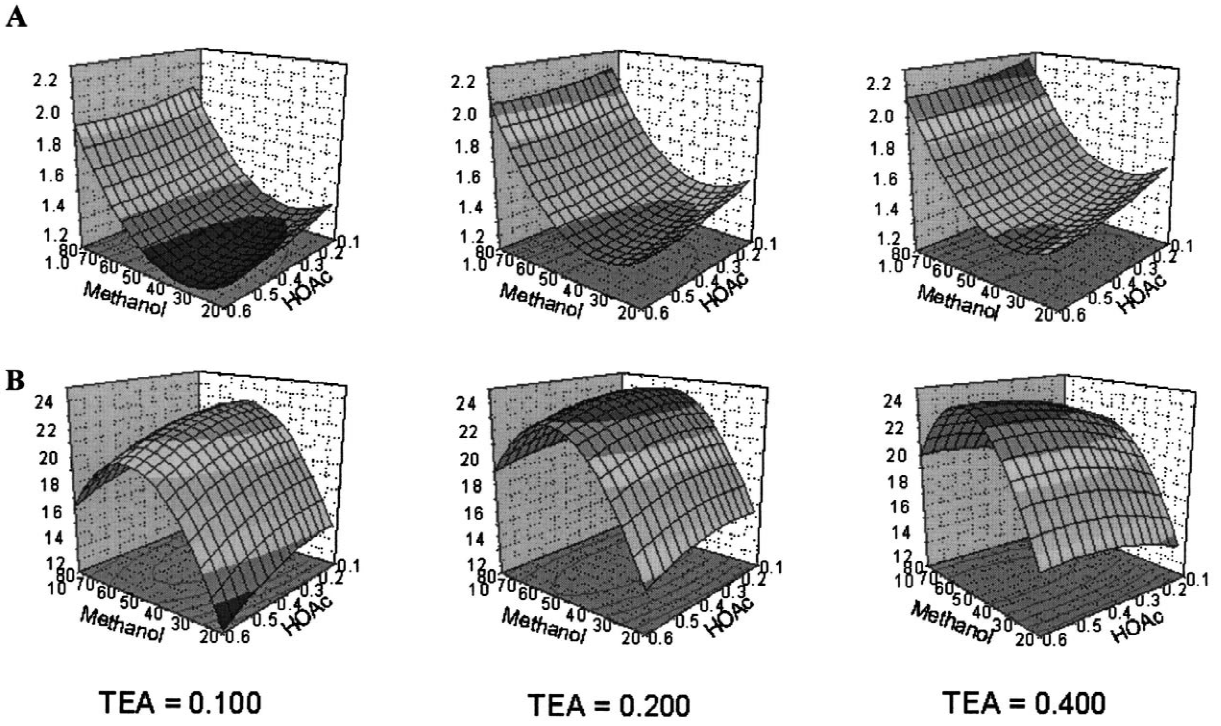

Fig. 7. Response surfaces generated from the data in Table 2 for (a) resolution and (b) efficiency by the Modde 4.0 statistical software for the chiral separation of thalidomide in the polar organic mode.

in value at higher MeOH content. It was found that at higher MeOH concentrations longer column equilibration times were required, thus a value of 80% was not exceeded. The effects of acid/base concentration are less dramatic where slightly higher resolution values may be obtained at higher base and lower acid contents for this analyte. The results for the efficiency response are a little more difficult to interpret since an efficiency maximum is predicted to occur at approximately 55% MeOH (Fig. 7b). Once again the effects of acid/base concentration are small with an optimum occurring when these are in equal concentration.

These conditions were examined experimentally but no increase in efficiency was obtained. A decrease in resolution was obtained, however, as predicted from the resolution response model. It may be possible that column deterioration over time resulted in this ''misleading'' generated optimum MeOH content at 55%. It may also explain the relatively low R^2 values for the efficiency model (0.86). It was noted that although column efficiency values of 120 000 plates meter^{-1} were routinely obtained and run almost constantly for two months, a drop in efficiency (ca. 25%) was observed for thalidomide enantiomers when examined under the exact conditions at the start of the study and after the chemometric and repeatability run experiments. This drop in this time scale may indicate the lifetime of these in-house prepared vancomycin columns for CEC.

Repeatability data for the thalidomide separation in the polar organic mode were calculated. RSD values ($n=7$) for migration time, resolution, area and efficiency were found to be 0.4%, 0.9% 4.0% and 2.0%, respectively. These values can be considered acceptable for preliminary data on a novel CSP prepared for CEC.

Throughout the course of the study several Ohm's plot curves were carried out to determine if Joule heating effects were contributing to enhanced or decreased chromatographic response values. Typical

values for current generated when using the polar organic mode (ca. 22 μamps when 30 kV is applied to a 33.7 cm×75 μm column with MeOH/MeCN/HOAc/TEA, (80:20:0.2:0.2, v,v,v,v) were generally higher compared to those generated when using reversed-phase conditions (ca. 2.8 μamps on the same column and conditions with MeCN/0.1% TEAA (pH 6.5), 30:70, v/v). Considering these low values coupled with linear Ohm's plots it was concluded that Joule heating was not taking place.

4. Conclusions

This study has demonstrated the feasibility employing silica diol packed capillaries for evaluation in CEC and subsequent in-situ immobilisation of the vancomycin chiral selector for enantiomeric separations. Silica diol CEC columns were packed repeatably and shown to be selective for a biphenyl-pyrene test mix in the reversed-phase mode. Acceptable efficiency values with a 5 μm LiChrospher® 100 Å diol phase were obtained throughout the study resulting in acceptable efficiency values, ca. 100 000 plates meter^{-1} at a linear velocity of 0.75 mm/s. The influence of pore size in CEC for two diol materials was also examined prior to any immobilisation of chiral selector and found to be much more influential. The efficiency values obtained in CEC with a 10 μm LiChrospher® 1000 Å diol silica were compared to those obtained with the 100 Å material described above and despite having twice the particle size, reduced plate height values were four times lower. These findings are of course not fundamentally novel since similar results have been shown in CEC for ODS packed columns [32] but have not been shown previously on packed diol capillary columns.

Enantioselectivity was obtained for thalidomide in the reversed-phase mode on a vancomycin CSP prepared by a relatively simple immobilisation procedure [29]. This chiral separation which offered ca. 80 000 plates meter^{-1} was found to be repeatable from run-to run but also from column-to-column. A number of neutral, basic and acidic analytes were subsequently screened on this CSP for enantioselectivity in the reversed-phase mode but with little success. This was overcome, however, by employing the polar organic mode where enantioselectivity was obtained for ten racemic analytes. Enantioselectivity was only obtained, however, for those analytes that contained basic functionalities with the exception of the weak acid thalidomide, that incidentally also has a secondary amine function. Efficiency values of approximately 45 000 and 120 000 plates meter^{-1}, were obtained for the β-adrenergic blocking agents and thalidomide on this vancomycin CSP in the polar organic mode. A small chemometric design was carried out to evaluate the polar organic mobile phase components which indicated that organic modifier content ratio, MeOH/MeCN had a significant effect on both resolution and efficiency. It was also shown that for this weak acid, thalidomide, although the effects of TEA/HOAc had a lower effect than modifier ratio, higher resolution values were obtained at higher concentrations of TEA.

Further studies are now underway to evaluate these preliminary data indicating high efficiency and resolution values for both the diol silica phase and the vancomycin CSP further. It is clear that the poor results obtained for this CSP in the reversed-phase mode need to be probed even further to gain an in-depth understanding of the enantioselective mechanism taking place for thalidomide, so that it may be used advantageously for many other analytes.

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