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Research Article

Two Synthetic Methods for Preparation of Chiral Stationary Phases Using Crystalline Degradation Products of Vancomycin: Column Performance for Enantioseparation of Acidic and Basic Drugs

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Abstract. Two chiral stationary phases (CSPs) based on crystalline degradation products (CDPs) of vancomycin by using different synthetic methods were prepared and compared. Crystalline degradation products of vancomycin were produced by hydrolytic loss of ammonia from vancomycin molecules. Performances of two chiral columns prepared with these degradation products were investigated using several acidic and basic drugs as model analytes. Retention and resolution of these analytes on the prepared columns, as two main parameters, in enantioseparation were studied. The results demonstrated that the stationary phase preparation procedure has a significant effect on the column performance. The resolving powers of prepared columns for enantiomers resolution were changed with the variation in vancomycin-CDP coverage on the silica support. Elemental analysis was used to monitor the surface coverage of silica support by vancomycin-CDP. The results showed that both columns can be successfully applied to chiral separation studies.

KEY WORDS: chiral stationary phase; enantioseparation; synthetic method; vancomycin-CDP.

INTRODUCTION

Optical isomers often possess different biological properties which lead to therapeutic or toxic effects on the organisms. Therefore, enantiomeric separations are very important, especially in the pharmaceutical and agricultural industries. Among all the chiral separation techniques, high-performance liquid chromatography (HPLC) is known as an appropriate method, due to its availability, simplicity, cost, and reproducibility. Most of HPLC methods are equipped with a chiral column (CS) filled with a chiral stationary phase (CSP) for enantimeric separations.

The use of macrocyclic antibiotics as CSP in HPLC was first introduced by Armstrong in 1994 (1). These CSPs have been applied to enantiomer separation of a wide variety of compounds (2–8). Vancomycin is a glycopeptide antibiotic with various functional groups such as hydroxyl, carboxyl, amine and aromatic groups, and three macrocyclic rings, which provide a variety of interaction centers for chiral recognition (9–12). Due to these properties, vancomycin is used as CSP and chiral mobile phase additive (CMPA) for enantioslective separation of various analytes using HPLC and capillary electrophoresis (CE) (13–19). Vancomycin has two crystalline degradation products (CDPs), namely, CDP-1-M and CDP-1-m (Fig. 1), which are formed by hydrolytic loss of ammonia (19). The CDPs are structurally similar to vancomycin, with two carboxyl groups, but they are biologically inactive (20). Determination of vancomycin and their CDPs by HPLC and pyrolysis-mass spectrometric methods has been reported previously (20,21). In the previous works, the ability of vancomycin-CDP chiral stationary phase in enantioseparation of some drugs has been established (22–25).

It is demonstrated that, with changing the chiral selector coverage on support material of CSPs, the stereoselective interactions of CSP with analytes and enantiomeric resolutions are altered (26). The linkage of the CS to silica support is one of the most important techniques to prepare CSPs with various coverage degrees of chiral selectors. Usually, the higher coverage of CS on the silica support results in increased interactions between the CSP and, consequently, improves the retention factors and enantiomeric resolutions of analytes.

In the present work, a new method for covalently bonding of vancomycin-CDP chiral stationary phase to silica gel was developed. Meanwhile, a second column was also prepared according to the previous reports (22–25). Finally, the applicability of the two prepared chiral columns for enantioseparation of several selected drugs by HPLC was investigated.

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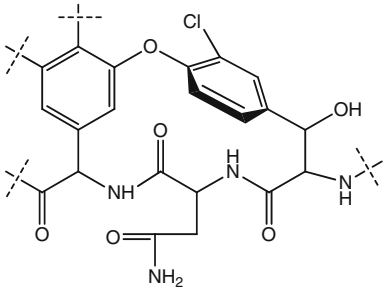
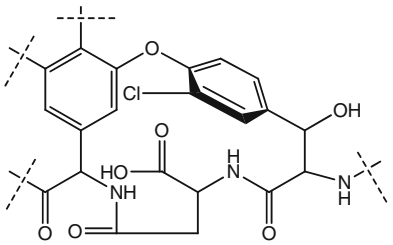
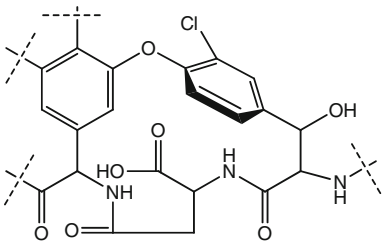


vancomycin



CDP-1-M



CDP-1-m

Fig. 1. Structures of vancomycin and its degradation products

EXPERIMENTAL

Chemicals and Reagents

The racemic analytes including atropine, amlodipine, baclofen, ibuprofen, mandelic acid, and phenylalanine were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Vancomycin was obtained from Zakaria Pharmaceutical Co. (Tabriz, Iran). 3-Aminopropyl silica gel (3-APSG) (Irregular POLYGOSIL® 60 NH_2, 5 μm, 350 m^2/g) was purchased from Macherey-Nagel (Düren, Germany). 1,6-hexamethylene diisocyanate (HMDI), n-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), dimethylformamide (DMF), pyridine, toluene, methanol, tetrahydrofuran (THF), triethyl amine, acetic acid, acetone, triethylammonium acetate (TEAA), chloroform, and dichloromethane were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC grade and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) membrane (Millipore, USA) before use.

Apparatus and Chromatographic Conditions

The IR spectra were recorded on a Fourier-transform infrared spectrometer (FT-IR, BRUKER, model TENSOR 27, Germany). Ten milligrams of samples were mixed with 1 g potassium bromide (KBr), and the mixture was transferred to a mortar and grind to a fine powder. Finally, a small amount of fine powder was compressed into a thin transparent pellet using a hydraulic press.

A Knauer HPLC instrument consisted of a low pressure HPLC pump (K-1001), a solvent organizer (K-1500), a photodiode array (PDA) detector (K-2800), and a computer-controlled system with ChromGate® software was used. The injection of samples was performed using a manual injector (Rheodyne model 7725i) with an injection volume of 20 μL. All chromatographic experiments were performed in an isocratic mode. The mobile phase flow rate was 1.0 mL min^{-1}. Experiments were performed at room temperature. The mobile phase was filtered and degassed through a 0.45 μm PTFE membrane filter.

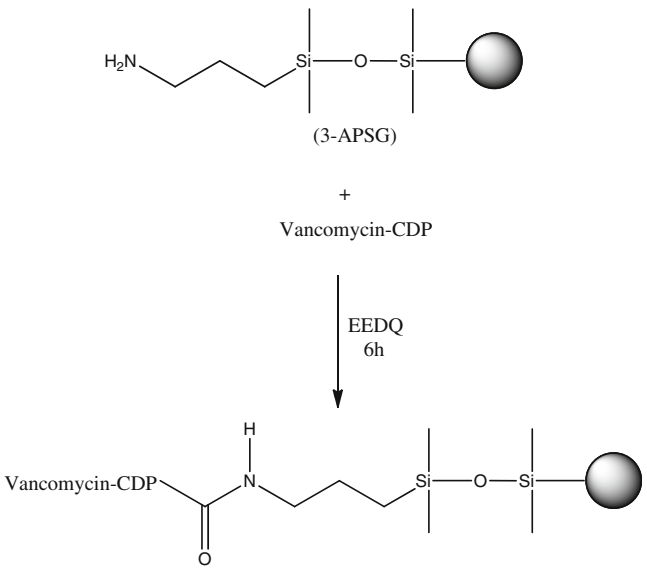


Fig. 2. Schematic diagram for preparation packing material of column A

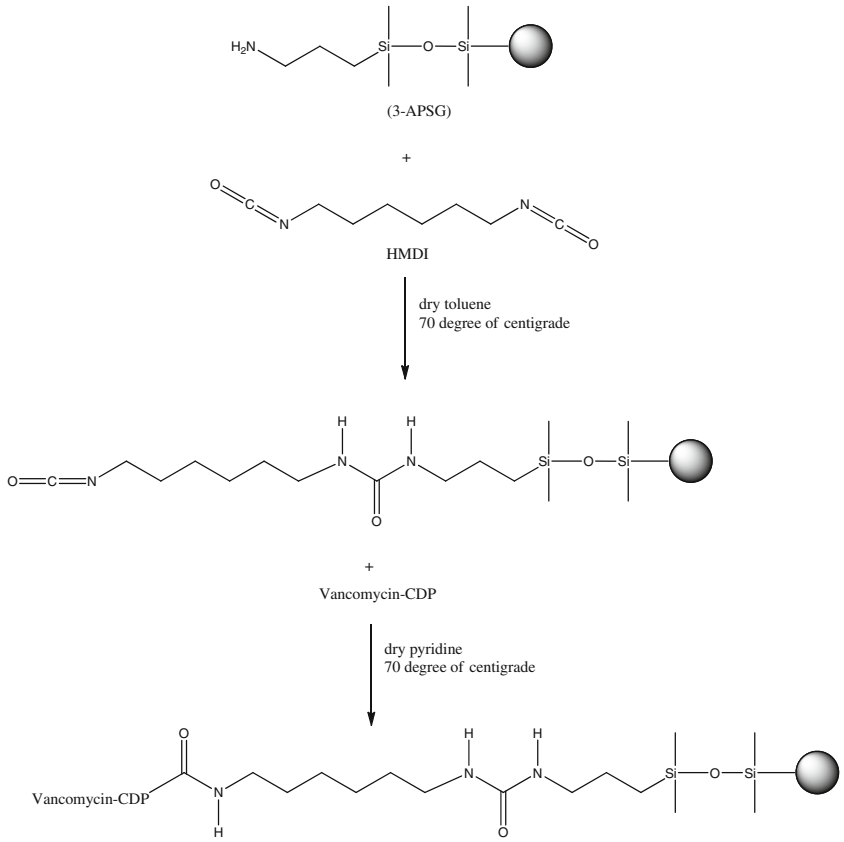


Fig. 3. Schematic diagram for preparation packing material of column B

Preparation of Chiral Stationary Phases and Columns

Conversion of Vancomycin to CDP-1-M and CDP-1-m

The crystalline degradation products of vancomycin were prepared by dissolving 2.0 g of vancomycin hydrochloride in 30 mL water and adjusting the pH to 4.2 by addition of sodium hydroxide solution (1.0 M). The solution was refluxed at 60–70°C for 40 h and then cooled to room temperature. The obtained solids were isolated by filtration and washed with water and dried (21).

Preparation of Packing Material of Column A

Macrocyclic antibiotics containing amine, hydroxyl, or carboxylic acid functional groups can be linked to silica gel by different approaches (1,5,6). Vancomycin-CDP contains one or more of these functional groups. In a typical reaction, 4.0 g dry 3-APSG was slurried in anhydrous DMF. Then, vancomycin-CDP (1.0 g) and EEDQ as dehydrating agents were added to the mixture. After 6 h, the CSP material was isolated by filtration and washed with methanol and aqueous methanol. The obtained packing material was dried. The schematic diagram for preparation packing material of column A is shown in Fig. 2 (22).

Preparation of Packing Material of Column B

Column B was prepared according to the method described by D'Acquarica (26). Briefly, 2.5 mL of HMDI was added to ice-bath cooled slurry of dry 3-APSG (4.0 g in 50 mL dry toluene). The mixture was removed from ice-bath and heated at 70°C for 2 h. Then, the mixture was cooled to room temperature and the liquid phase was filtered under a nitrogen atmosphere. The excess HMDI was removed from activated 3-APSG by washing with 10 mL dry toluene. A suspension of 1.0 g vancomycin-CDP in 100 mL dry pyridine was added to the activated 3-APSG, and then the mixture was heated at 70°C for 12 h with continuous stirring. After cooling, the CSP was filtrated and washed with 50 mL portions of pyridine, water, methanol, and dichloromethane, respectively. Finally, the obtained packing material was dried under vacuum. The schematic diagram for preparation packing material of column B is shown in Fig. 3.

Packing of Columns A and B

The prepared packing materials (5 μm particles) were slurry packed into two stainless steel columns (250 × 4.6 mm) by means of a Knauer K-1900 pneumatic pump. A mixture of

Table I. Results of Elemental Analysis of Two Synthesized CSP and Their Solid Supports

Material	C (%)	H (%)	N (%)
3-APSG	2.23	0.8	0.82
3-APSG-HMDI	7.79	1.48	2.65
Column A	3.72	0.94	1.07
Column B	16.73	2.27	3.98

acetone/chloroform (1:1 *v/v*) and ethanol was used as slurring and pressurizing agents, respectively.

RESULTS AND DISCUSSION

The elemental analysis results for 3-APSG and two synthesized CSPs are shown in Table I. According to these results, the two columns have different amounts of CSP coverage so that column B possesses a higher coverage than column A. Therefore, it is expected that the two columns show different behaviors and performances for enantiomeric separations.

In order to investigate the connection of vancomycin-CDP to modified 3-APSG with HMDI, the FT-IR spectra before and after the reaction of activated 3-APSG with vancomycin-CDP were recorded (Fig. 4). The new proposed mechanism for preparation of column B was approved by disappearance of the isocyanate groups, −N=C=O (2200–2300 cm^{-1}) and appearance of the ureidic group, −NH–CO–NH− (1655 cm^{-1}), in the FT-IR spectra (Fig. 4) (26). During the synthesis, the free amino groups of vancomycin-CDP are reacted with isocyanate and, consequently, additional linkages between glycopeptides alcoholic or phenolic hydroxyls with isocyanate groups are created. Therefore, ionizable groups of the prepared column may be different from the commercial columns, which are expected to lead to different chromatographic behaviors.

As it is quite clear from Figs. 1 and 2, the chain length of spacer in column B is longer than that in column A. This phenomenon reduces the steric hindrance between vancomycin-CDP molecules for reaching the silica surface. Therefore, an increased number of molecules find the opportunity to link to the silica support which results in increased CSP coverage of column B compared to that of column A (see Table I).

The resolving power of the prepared vancomycin-CDP stationary phases was investigated by the analyses of six different racemic compounds, as depicted in Table II. The chromatographic conditions used for separation of these

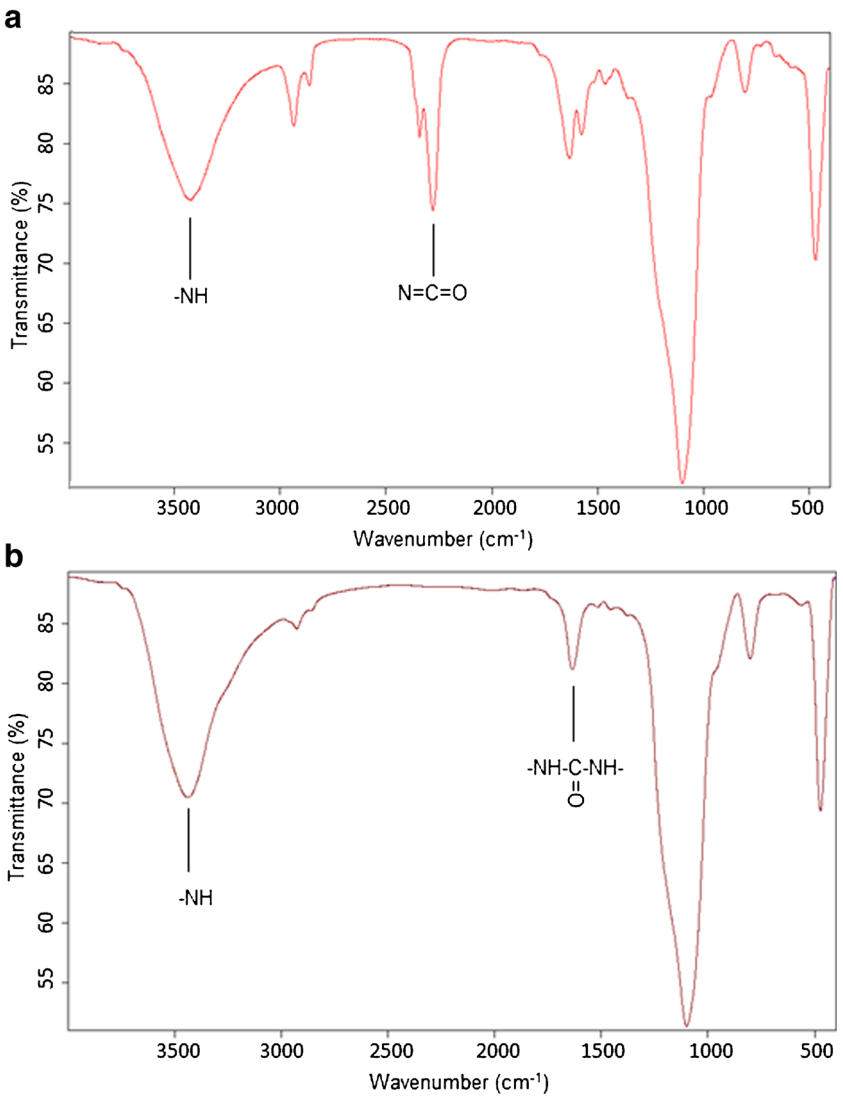


Fig. 4. FT-IR spectra of HMDI linked to 3-APSG (**a**) and activated 3-APSG linked to vancomycin-CDP (**b**)

Table II. Chromatographic Conditions Used for Separation of Selected Enantiomers on Vancomycin-CDP Stationary Phases

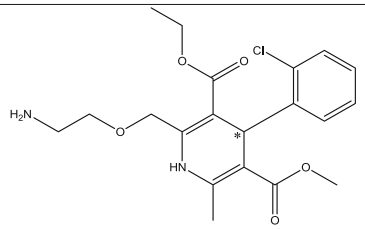
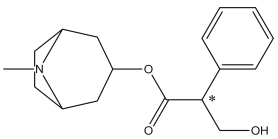
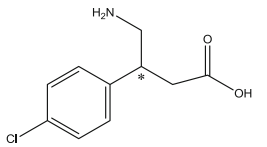
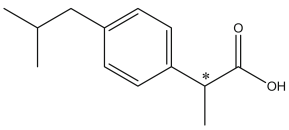
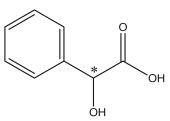
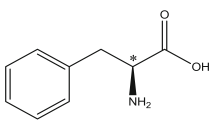
Analyte	Structure	Detection wavelength (nm)	Eluent for two columns
Amlodipine		210	100% methanol°+°0.1% NH_4TFA
Atropine		235	100% methanol°+°0.1% NH_4TFA
Baclofen		220	100% methanol°+°0.1% NH_4TFA
Ibuprofen		254	THF/20 mM sodium citrate **in water**, pH°=°6.3 (10/90)
Mandelic acid		254	Methanol/TEAA pH°=°6.5 (15/85)
Phenylalanine		210	100% methanol°+°0.1% NH_4TFA

Table III. Resolving Power of Two Columns on Enantioselectivity and Resolution of Acidic and Basic Drugs

Analyte	Columns											
	A						B					
	(3-APSG + CDP)						(3-APSG + HMDI + CDP)					
	t_{R1}	t_{R2}	k_1	k_2	α	R_S	t_{R1}	t_{R2}	k_1	k_2	α	R_S
Racemic amlodipine	2.10	3.32	1.10	2.32	2.11	3.12	12.87	18.57	11.87	17.57	1.48	2.98
Racemic atropine	1.48	2.03	0.48	1.03	2.15	2.75	2.50	2.50	1.50	1.50	1	N/R
Racemic baclofen	8.42	9.39	7.42	8.39	1.13	0.50	3.42	3.62	2.45	2.62	1.07	0.25
Racemic ibuprofen	2.10	2.52	1.10	1.52	0.98	0.84	2.80	3.40	1.80	2.40	1.34	2.4
Racemic mandelic acid	6.93	7.42	5.93	6.42	1.08	0.98	5.80	7.40	4.80	6.40	1.34	1.78
Racemic phenylalanine	2.38	3.30	1.38	2.23	1.62	2.10	6.08	7.58	5.08	6.58	1.33	3.05

N/R not resolved

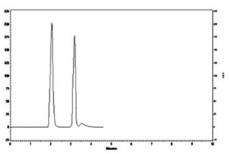
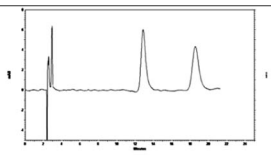
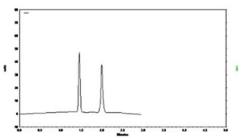
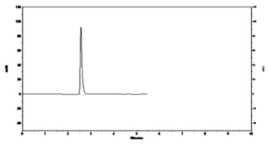
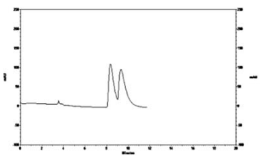
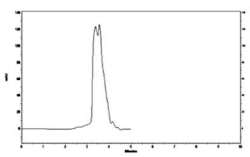
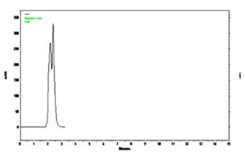
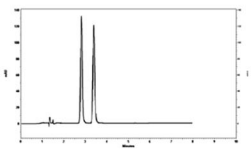
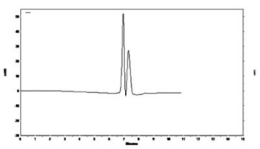
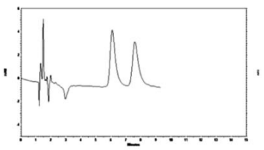
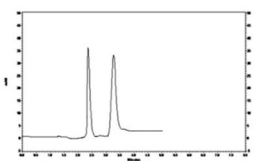
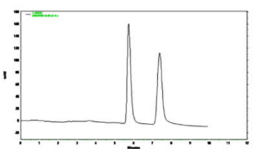
selected racemates are also summarized in Table II. The obtained chromatographic data including retention times (t_{R1} and t_{R2}), retention factors (k_1 and k_2), selectivity factor (α), and resolution (R_S) for analysis of the analytes under the mentioned conditions are listed in Table III.

It is worth mentioning that, in the case of R_S values of 1.5 and more (see Table III), the two enantiomers can be completely separated to the baseline. Using column A, enantiomers of amlodipine, atropine, and phenylalanine were separated completely. Whereas column B is enable to completely separate the enantiomers of amlodipine, ibuprofen, mandelic acid, and phenylalanine. It is reported that the sp^2 hybridized carbons directly connected to the chiral center would greatly enhance the enantiorecognition of a molecule (27). Among these analytes, amlodipine has three sp^2 hybridized carbons attached to its chiral center. While ibuprofen, mandelic acid, and atropine possess two sp^2 hybridized carbons, baclofen and phenylalanine have only one of these.

The polar ionic mode is also applicable to all molecules as they have at least one ionizable group on or near the chiral center and one additional functional group somewhere in their structures. Usually, basic compounds such as amlodipine demonstrate more selectivity in this mobile phase. In this study, except with ibuprofen and mandelic acid, unbuffered mobile phases were used for other selected drugs. In the working pH range of the column (*i.e.*, $3.5 < pH < 8$), the carboxylic acid moieties of CSP are in their anionic form (COO^-). While, over the pH range of 4–7, the amino groups of CSP are protonated, which produce positively charged sites in its structure. The main factors influencing the chromatographic behavior of acidic drugs such as ibuprofen and mandelic acids are interactions of their anionic forms with the polar groups of the vancomycin-CDP selectors together with the corresponding and π–π interactions.

Performances of the two columns prepared for separation of the selected drugs are shown in Table IV. As is obvious from Table IV, the two columns possess different

Table IV. Efficiencies of Two Columns in Separation of Enantiomers of Several Drugs

Analyte	Column A	Column B
Amlodipine		
Atropine		
Baclofen		
Ibuprofen		
Mandelic acid		
Phenylalanine		

resolving powers for the same racemate. This phenomenon may be related to the amount of vancomycin-CDP loading on the silica surface, which leads to different interactions between analytes and CSP. Table IV shows that the resolving power of column B for ibuprofen and mandelic acid is better than that of column A. On the other hand, atropine and baclofen were better resolved using column A. On the other hand, the size of chiral selector molecules in both columns is smaller than that in commercially available vancomycin column, which leads to high coverage of chiral selector on the silica support. Also, the chiral selector molecules are degradation products of vancomycin which prepared under the high temperatures. Therefore, these columns can tolerate a wider pH range and higher temperatures than the commercial vancomycin stationary phase.

Maximum loading capacities for the two columns proposed are 50 μL. The resolving powers of column A and column B were deteriorate after 50 and 80 injections, respectively. The prepared columns with the same procedure were examined for reproducibility test. The relative standard deviations (RSDs) for retention times using racemic amlodipine were less than 5.5%.

CONCLUSION

Crystalline degradation products (CDPs) of vancomycin have structures similar to vancomycin with small differences. Vancomycin-CDPs have two carboxylic acid groups whereas vancomycin has one only. Vancomycin-CDP has been linked to the surface of silica using two different methods to produce different CSPs. Several racemic compounds can be separated on these CSPs, which could have potential applications in enantiomeric separation of chiral compounds. The results showed that the synthetic procedure of CSP can influence the column performance.

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