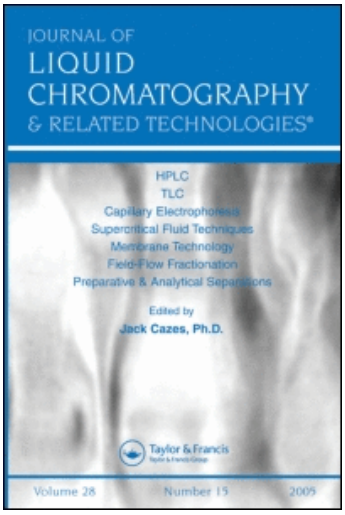

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Jing Yuan[a]; Junjuan Xu[b]; Wenqiang Niu[a]; Qingxiang Zhao[a]; Husheng Yan[a]; Xiaohui Cheng[a]; Binglin He[a]
[a] The State Key Laboratory of Functional Polymer Materials, for Adsorption and Separation, Institute of Polymer Chemistry, Nankai University, Tianjin, P. R. China [b] Tianjin College of Traditional Chinese Medicine, Tianjin, P. R. China

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AFFINITY ADSORBENTS WITH D-ALANINE AND D,L-ALANINE AS LIGANDS FOR VANCOMYCIN GROUP ANTIBIOTICS

Jing Yuan,[1] Junjuan Xu,[2] Wenqiang Niu,[1] Qingxiang Zhao,[1] Husheng Yan,[1,*] Xiaohui Cheng,[1] and Binglin He[1]

[1] The State Key Laboratory of Functional Polymer Materials, for Adsorption and Separation, Institute of Polymer Chemistry, Nankai University, Tianjin, P. R. China
[2] Tianjin College of Traditional Chinese Medicine, Tianjin, P. R. China

ABSTRACT

Two affinity adsorbents for the vancomycin group antibiotics were prepared by immobilizing D-alanine and D,L-alanine, respectively, onto crosslinked polyacrylamide resin through the Mannich reaction. The adsorption of N-demethylvancomycin on the adsorbents was studied. The results showed that the adsorption capacity of the D-alanine-immobilized adsorbent was higher than that of the D,L-alanine-immobilized one when the immobilization capacities were the same, indicating the affinity of the immobilized D-alanine for N-demethylvancomycin was greater than that of the immobilized L-alanine.

Chromatographic adsorption and desorption of N-demethylvancomycin on a column packed with D,L-alanine-immobilized

* Corresponding author. E-mail: yanhs@public.tpt.tj.cn

adsorbent were carried out. The saturation adsorption capacity was ~0.45 mmol/g (dry resin) or ~0.064 mmol/mL (swollen resin) when the initial concentration of N-demethylvancomycin was 6.62 mM. The adsorbed N-demethylvancomycin on the column was eluted using 0.1 M HCl in water/ethanol (7/3, v/v) as eluant.

There were two peaks in the elution curve. The first peak (with lower elution volume) may have resulted from the N-demethylvancomycin being adsorbed by the immobilized L-alanine, while the second peak may correspond to the N-demethylvancomycin adsorbed by the immobilized D-alanine. The area ratio of the first peak to that of the second one was ~1/4, indicating that the immobilized D-alanine adsorbed more N-demethylvancomycin than the immobilized L-alanine.

INTRODUCTION

The vancomycin group antibiotics is a clinically important group of glycopeptides against Gram-positive bacteria.(1,2) Among the antibiotics, vancomycin and teicoplanin have been clinically used in America and Europe, and N-demethylvancomycin, which is a methyl group less and more active against bacteria than vancomycin, has been clinically used in China.(3) The mode of action of the group antibiotics is based on their ability to bind to the nascent cell wall mucopeptide precursors of Gram-positive bacteria, terminating in tripeptide -L-Lys-D-Ala-D-Ala, to block the cell wall synthesis.(1,4,5)

According to the binding between vancomycin group antibiotics and the bacterial cell wall, affinity adsorbents for the antibiotics have been prepared by immobilizing cell wall analogues to solid supports. Perkins and Nieto(6) linked N^a-Ac-L-Lys-D-Ala-D-Ala onto CM-cellulose and found that adsorption of vancomycin on the support mimics its natural binding to cell wall preparations. Corti and Cassani(7) prepared affinity adsorbent for vancomycin group antibiotics by immobilizing D-Ala-D-Ala onto activated CH-Sepharose 4B. This adsorbent bioselectively binds glycopeptide antibiotics teicoplanin, vancomycin, and restocetin A, and has been used to purify teicoplanin from fermentation broth. Folena-Wasserman and coworkers(8) synthesized affinity adsorbents by immobilizing cell wall peptides onto an N-hydroxysuccinimide-activated agarose support. All of these affinity adsorbents were made by linking expensive cell-wall analogue peptides onto expensive activated natural polymeric supports.

In previous papers,(9,10) we synthesized cell wall analogue-immobilized affinity adsorbents by building the amino acids one by one onto synthetic polymeric supports, based on the principle of the solid phase peptide synthesis. Although the synthesis cost is relatively low, these affinity adsorbents are still too

expensive for practical application. In this paper, we report on affinity adsorbents with very low cost for vancomycin group antibiotics.

EXPERIMENTAL

Materials

N-Demethylvancomycin was obtained from North China Pharmaceutical Corporation as hydrochloride salt. Acrylamide (AR) and N,N'-methylene bisacrylamide (AR) were purchased from Beijing Chemical Plant. Potassium persulfate (AR), which was recrystallized before use, was purchased from Tianjin Chemical Plant. Aqueous formaldehyde (36%, AR) was purchased from Ji-nan Organic Chemical Plant. D-alanine (AR) and D,L-alanine (AR) were purchased from Dongfeng Biotechnology Co. All other reagents were analytical grade.

Synthesis of Crosslinked Polyacrylamide

A solution containing 9.0 g of acrylamide, 1.0 g of N,N'-methylene bisacrylamide, 0.1 g of potassium persulfate, and 50 mL of water was suspended in a solution of 160 mL of chlorobenzene, 90 mL of toluene, and 14 g of span-85. Under nitrogen atmosphere and stirring with controlled speed, a drop of N,N,N′,N′-tetramethylethylenediamine was added to the mixture. The stirring was continued for 50 min at ambient temperature and 10 min at 70°C. The resulting polymer was filtered off, washed 3 times with toluene and 3 times with acetone, and then dried in air. The polymer beads passing through sieves of 30-60 mesh were used in the following experiments.

Synthesis of Affinity Adsorbents

In a 100 mL three-neck round-bottom flask, 4.0 g of polyacrylamide described above, was suspended in 20 mL of 0.1 M sodium phosphate buffer (pH 10.5). To this was added 4.9 g of 36% aqueous formaldehyde. The mixture was stirred and heated at 40°C for 1 hr. Then, 20 mL of 0.1 M sodium phosphate buffer (pH 10.5) containing 5.7 g of D,L-alanine and 1.5 g of sodium hydroxide was added. The temperature of the mixture was raised to 70°C and remained for 2.5 hr. The resulting resin, D,L-alanine-immobilized adsorbent, was washed thoroughly with water and dried.

D-alanine-immobilized adsorbent was prepared similarly using D-alanine instead of D,L-alanine.

Determination of Loading Capacities of Alanine on the Adsorbents

The adsorbent samples (~0.2 g) were suspended in 25.0 mL of standard 0.1 M NaOH. After 24 hr standing, 10.0 mL of the supernatant was titrated with standard 0.1 M HCl using methyl orange as the indicator. The loading capacity was calculated according to the following equation:

$$\text{Loading capacity (mmol/g)} = (25N_{NaOH} - 2.5N_{HCl}V_{HCl})/W$$

where N_{NaOH} and N_{HCl} were concentrations (M) of the standard NaOH and standard HCl, respectively. V_{HCl} was the volume (mL) of standard HCl consumed in the titration. W was the weight (g) of the adsorbent sample.

Static Adsorption and Desorption

The adsorbent samples (~0.06 g) were suspended in 10 mL of ~6 mM N-demethylvancomycin in 0.1 M $H_2PO_4^-$ -HPO_4^{2-} buffer (pH 5.8) and the mixtures were shaken for 12 hr. The concentrations before and after the adsorption were determined by optical density measurements at 280 nm. The adsorption capacities were calculated from the concentration differences.

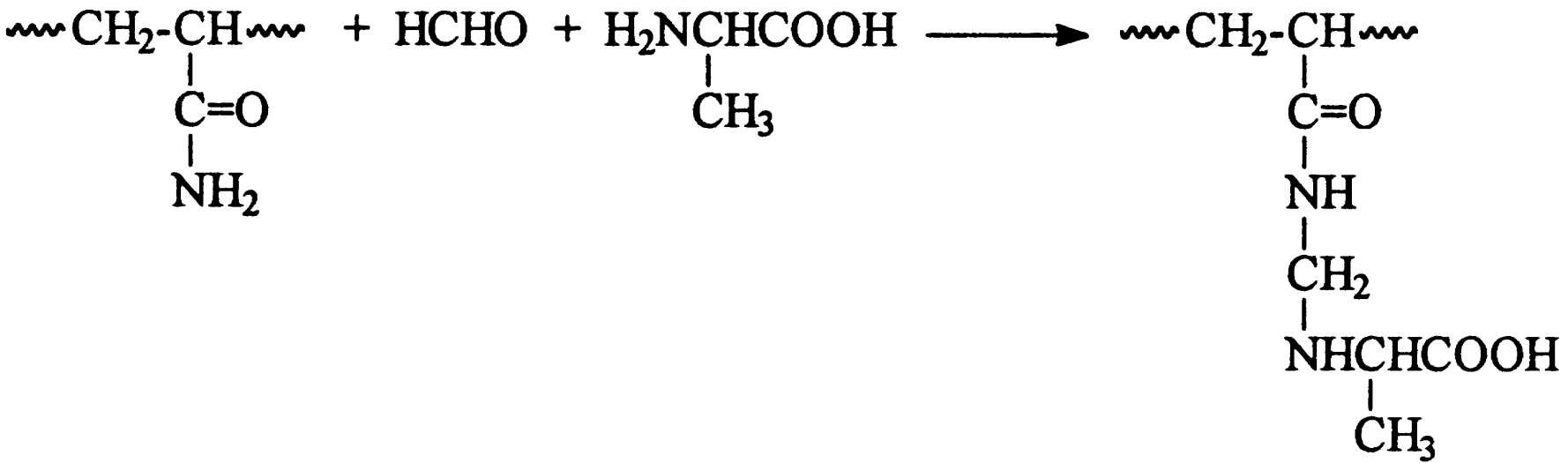
The above mixtures were filtrated. The adsorbents were quickly washed 2 times with the same buffer as above and then suspended in 25 mL of the desorption agent. The mixtures were shaken for 12 hr. The concentration of the antibiotic in the desorption agent was determined by optical density measurements at 280 nm and the desorption efficiency was calculated.

Dynamic Adsorption and Desorption

The D,L-alanine-immobilized adsorbent was swollen in 0.1 M $H_2PO_4^-$ -HPO_4^{2-} buffer (pH 5.8) and packed in a 250 × 5 mm, I.D., column (containing ~0.7 g of dry adsorbent) by the slurry method. N-demethylvancomycin (6.62 mM) in the same buffer was loaded at a flow rate of 2.5 mL/hr on the column that was pre-equilibrated with the buffer. The concentration of N-demethylvancomycin in the eluent was detected by the absorbance at 280 nm.

The column was washed with water in a flow rate of 30 mL/min for 15 min, then eluted with 0.1 M HCl in water/ethanol (7/3, v/v) at a flow rate of 2.5 mL/min. The concentration of the eluent was detected by the absorbance at 280 nm.

Most of the experiments in the study were carried out in single experiments. The errors were estimated by experiments to be within 10% for alanine-



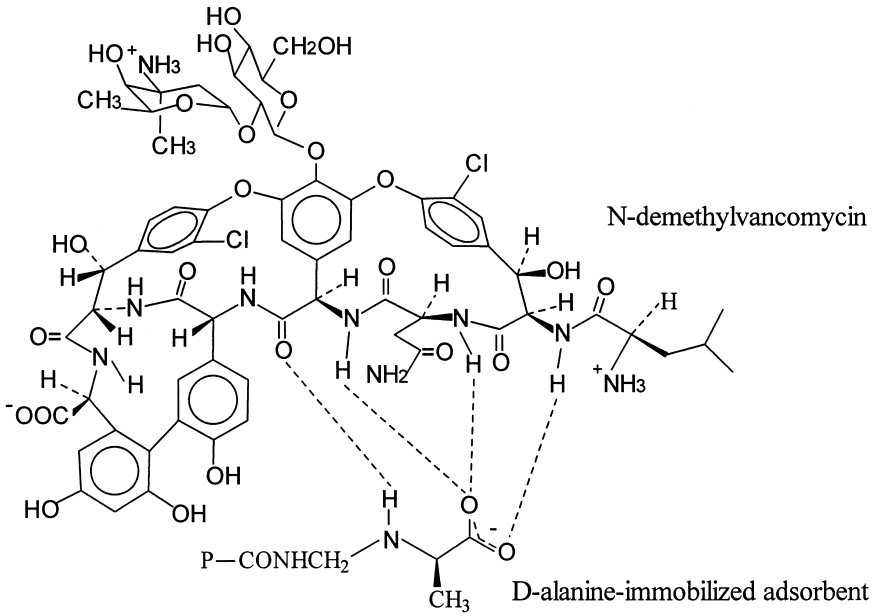


Figure 1. Model of interaction of N-demethylvancomycin with D-alanine-immobilized adsorbent. P in the adsorbent represents the polymeric matrix. The dotted lines represent hydrogen bonds.

with C-terminal L-alanine has no biospecific interaction with vancomycin group antibiotics, as mentioned above. As the ion concentration of the buffer was much higher than N-demethylvancomycin concentration in the solution, the non-specific interaction was much lower than the specific interaction of the D-alanine. As D,L-alanine is much cheaper than D-alanine, and for the consideration of a commercial application, only D,L-alanine-immobilized adsorbent was further studied.

Table 1. Ligand Contents and Adsorption Capacities for N-Demethylvancomycin

Ligand	Ligand Content (mmol/g)	Adsorption Capacity (mmol/g)
D-Ala	1.36	0.42
D,L-Ala	1.32	0.26

Table 2. Desorption Efficiency (%)

Desorption Agent	Desorption Efficiency (%)
0.1mol/L HCl	76.5
H_2O/C_2H_5OH (7/3)	8.9
0.1mol/L HCl/C_2H_5OH (7/3)	86.2
0.1mol/L Na_2CO_3/C_2H_5OH (7/3)	100

Figure 2 shows the effect of pH of the solution on the adsorption capacities of D,L-alanine-immobilized adsorbent for N-demethylvancomycin. The highest adsorption capacity occurred at about pH 6. This may be explained by the electrostatic interaction between the adsorbents and N-demethylvancomycin. As pK_a values of the N-terminal leucine ammonium of N-demethylvancomycin and the carboxyl group of the ligand of the adsorbent are ~9.7 and ~2.4, respectively, both have relatively high dissociation at pH~6.

The effect of equilibrium concentration of N-demethylvancomycin on the adsorption capacities of D,L-alanine-immobilized adsorbent is shown in Figure 3. The adsorption capacities increased with the increasing equilibrium concentration. Figure 4 shows the effect of concentrations of added salt in the solution

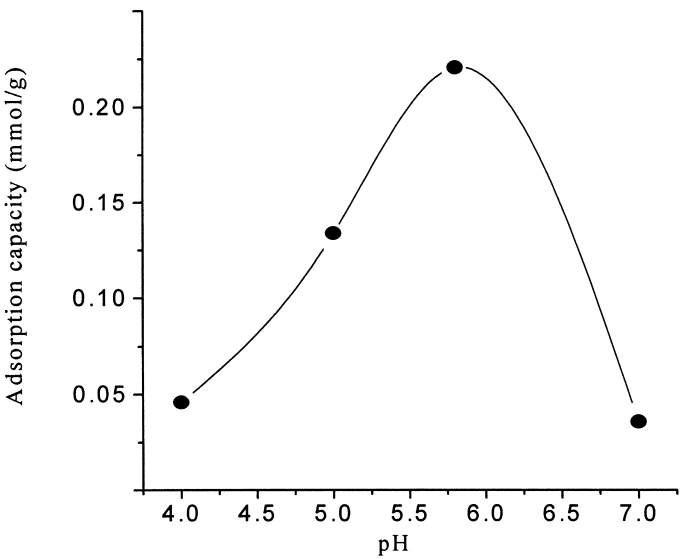


Figure 2. The effect of pH on adsorption capacity.

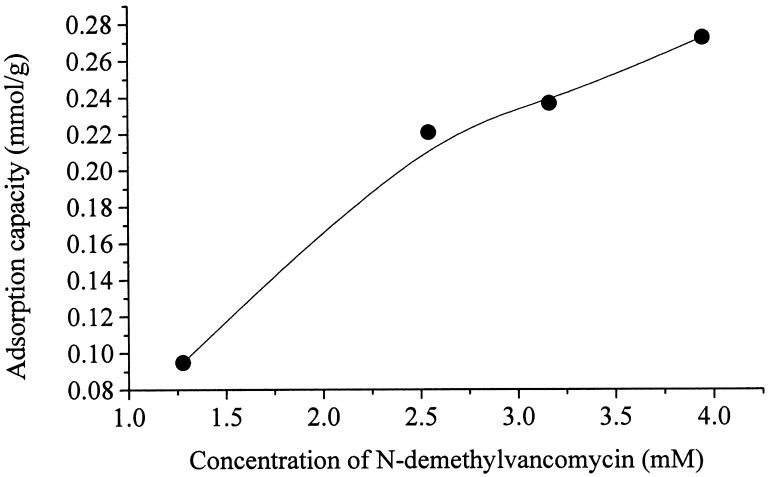


Figure 3. Plot of adsorption capacity against the equilibrium concentration of N-demethylvancomycin (pH 5.8).

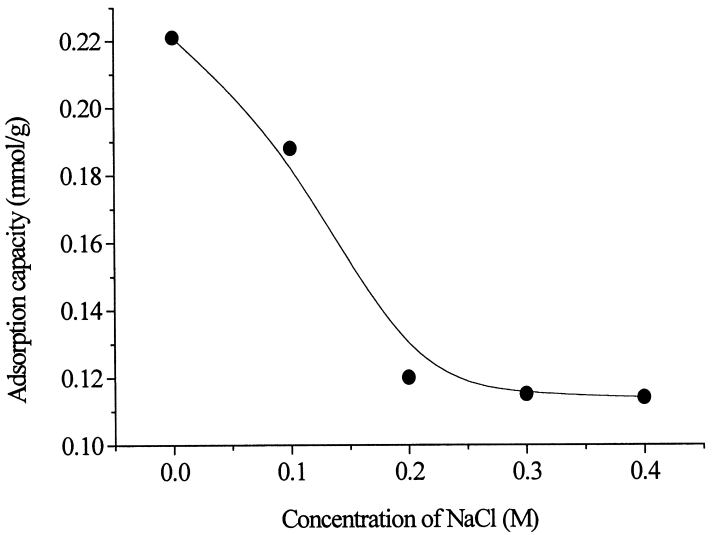


Figure 4. The effect of concentration of NaCl on adsorption capacity (pH 5.8).

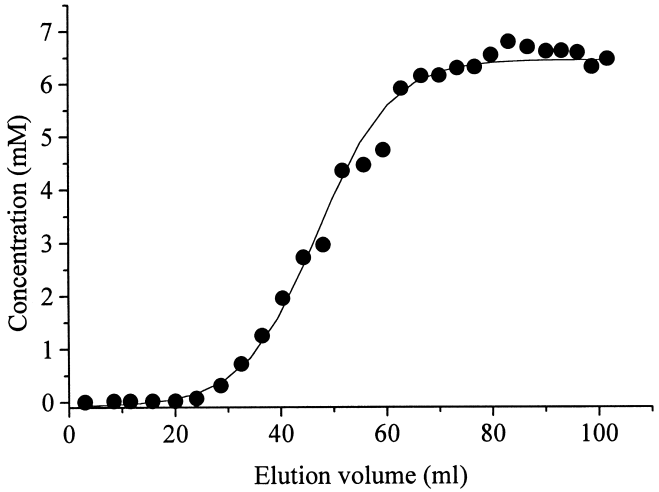


Figure 5. Adsorption profile of N-demethylvancomycin on D,L-alanine-immobilized adsorbent. (Column: 250 × 5 mm, I.D.; N-demethylvancomycin concentration: 6.62 mM; H: 5.8; flow rate: 2.5 mL/hr.)

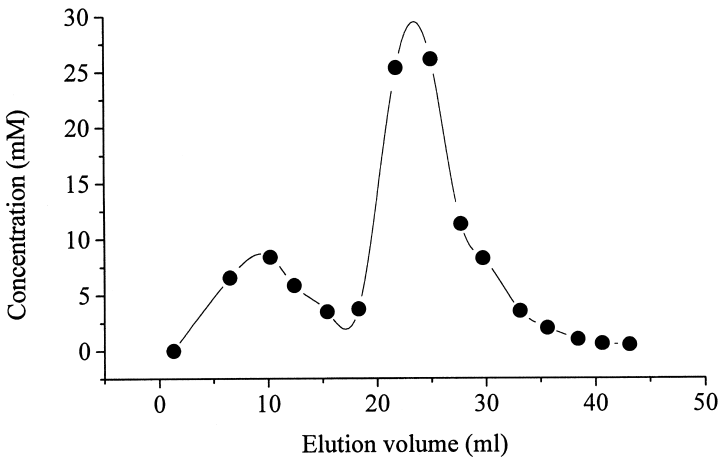


Figure 6. Elution graph (Eluant: 0.1 M HCl in water/ethanol (7/3, v/v); flow rate: 2.5 mL/min.

on the adsorption capacities. The adsorption capacities of the D,L-alanine-immobilized adsorbent decreased when the salt concentrations increased.

Three desorption agents were used to desorb the N-demethylvancomycin on the adsorbents, as shown in Table 2. The desorption efficiency of hydrogen chloride in aqueous ethanol was higher than that of aqueous hydrogen chloride or aqueous ethanol. The hydrogen chloride should weaken the electrostatic interaction and the ethanol should weaken the hydrophobic interaction.

As we had no fermentation liquor of vancomycin group antibiotics in hand, pure N-demethylvancomycin was used to test its chromatographic adsorption and desorption on a column packed with D,L-alanine-immobilized adsorbent. Figure 5 shows the elution concentration graph when N-demethylvancomycin was continuously loaded on the column. The leakage volume was ~30 mL and the saturation adsorption capacity was ~65 mL. The saturation adsorption capacity was calculated to be ~0.45 mmol/g (dry adsorbent) and ~0.064 mmol/mL (swollen adsorbent), respectively.

The column with saturation adsorption was eluted with 0.1 M HCl in water/ethanol (7/3, v/v), as shown in Figure 6. There were two peaks in the elution graph. The first peak with lower elution volume may be caused by N-demethylvancomycin adsorbed by the immobilized L-alanine and the second peak should corresponded to N-demethylvancomycin adsorbed by the immobilized D-alanine, because the affinity of the immobilized D-alanine for N-demethylvancomycin was greater than that of the immobilized L-alanine, as mentioned above. The area ratio of the first peak to the second one was ~1/4, indicating that the immobilized D-alanine adsorbed more N-demethylvancomycin than the immobilized D-alanine. This result is in agreement with the above conclusion.

CONCLUSION

D-alanine and D,L-alanine have been immobilized to crosslinked polyacrylamide to form affinity adsorbents for vancomycin group antibiotics. The immobilized D-alanine biospecifically binds N-demethylvancomycin, while the immobilized L-alanine does not. As the affinity adsorbents are very cheap and easy to synthesize, they may be applicable in vancomycin group antibiotic purification.

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