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Reversed Micellar Extraction of Vancomycin: Effect of pH, Salt Concentration, and Affinity Ligands

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ABSTRACT

Vancomycin was extracted from an aqueous feed solution into a reverse micellar solution of bis(2-ethylhexyl)sulfosuccinate sodium salt in isooctane. A low pH and salt concentration of aqueous feed solutions favors forward extraction. The backward extraction efficiency of vancomycin from reverse micelles into an aqueous phase, on the other hand, increases with pH. Affinity cosurfactants prepared by attaching a dipeptide D-alanyl-D-alanine or a racemic dipeptide DL-alanyl-DL-alanine to cholesteryl-chloroformate was employed for affinity reverse micellar extraction of vancomycin. The forward extraction efficiency increases significantly in the presence of an affinity cosurfactant. The recovery of vancomycin from fermentation broth with high selectivity was also achieved by employing this affinity cosurfactant.

Key Words. Vancomycin; Reversed micelles; Affinity reversed micellar extraction; D-Alanyl-D-alanine

INTRODUCTION

Glycopeptide antibiotic vancomycin was the first discovered in vancomycin group antibiotics in 1956 (1). More than 10 members of this group have since been reported. They all possess closely related chemical structures and are active against gram-positive bacteria. They continue to be clinically

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important therapeutic agents used for the treatment of infection owing to methicillin resistant *Staphylococcus sp.* (2), although in recent years an increase in resistance to them has been reported (3). In addition to its medical applications, vancomycin is now one of the more popular and successful chiral selectors used in separation science for the HPLC, TLC, and CE separation of optical isomers (4). The primary mode of antimicrobial action of vancomycin is inhibition of cell wall synthesis. Vancomycin binds very tightly to peptides that contain D-alanyl-D-alanine at the free carboxyl end and prevents the polymerization of peptidoglycan, which is vital for the existence of gram-positive bacteria, during cell wall synthesis (5). Based on this specific D-alanyl-D-alanine binding ability, the purification of vancomycin and several other glycopeptide antibiotics in the vancomycin group have been carried out by affinity chromatography (6, 7), affinity aqueous two-phase extraction (8, 9), and affinity ultrafiltration (10, 11) using D-alanyl-D-alanine as an affinity ligand.

Reversed micellar extraction systems have been the focus of much research activity in recent years due to their ability to selectively extract many biologically active materials such as enzymes (12, 13), antibiotics (14), and amino acids (15). The anionic surfactant sulfosuccinic acid bis(2-ethylhexyl) ester (AOT) and isooctane as an apolar solvent are usually employed to form reversed micelle system. In this reversed micelle system the polar head group (SO_3^-) of the AOT molecule is directed toward the interior of the micelle and forms a polar core which can solubilize water and the desired product; the lipophilic chains are exposed to the solvent isooctane. Electrostatic interaction is generally the main factor governing the partition behavior of the desired product between the aqueous feed phase and the reversed micelle phase. In other words, the extraction is based on the principle of liquid–liquid ion exchange. In order to increase the selectivity of reversed micelle extraction, a cosurfactant attached to a small affinity ligand can be incorporated into the reversed micelle to extract the product through affinity interaction. Separation of proteins using this affinity reversed micellar extraction has been investigated by several research groups (16–21).

In this work the extraction of vancomycin from aqueous buffers of various pH and salt concentrations into AOT–isooctane reversed micelles is studied. The backward extraction of vancomycin from reverse micelles into aqueous buffers is also studied. In order to increase the extraction capacity and selectivity, affinity reversed micellar extraction in which an affinity cosurfactant was added was employed. The affinity cosurfactant was prepared by attaching an affinity ligand (D-alanyl-D-alanine or DL-alanyl-DL-alanine) of vancomycin to cholesterol. The effect of affinity cosurfactant for improving the extraction efficiency of vancomycin is investigated. In addition, the recovery of vancomycin from fermentation broth using affinity reversed micellar extraction is also demonstrated.

MATERIALS AND METHODS

Materials

Vancomycin, D-ala-D-ala, DL-ala-DL-ala, L-ala, and cholesteryl chloroformate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) and isooctane were purchased from ACROS Chemical (New Jersey, USA). All other chemicals were of analytic grade.

Immobilization of Ligand in Reversed Micelles

Various ligands were dissolved in 300 μL of borate buffer (pH 10), and this ligand solution was mixed with 5 mL of a 0.1 M AOT solution of isooctane to be solubilized in the reversed micelles. Then 0.5 mL of cholesteryl-chloroformate (80 mg/mL) in isooctane was added to the reversed micelles solution of AOT and stirred continuously for 1 hour at room temperature. The cholesteryl group was introduced to the amino group of the ligand as shown in Fig. 1. The

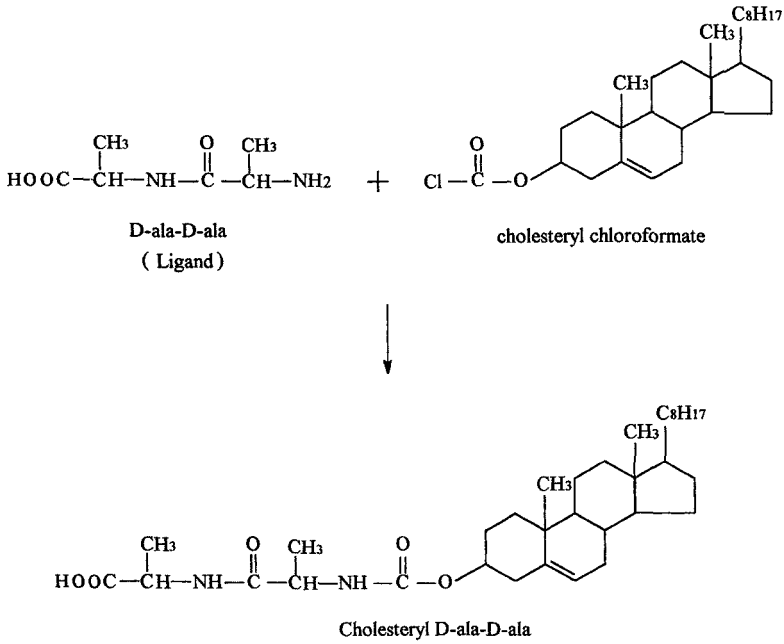

FIG. 1 Schematic representation of the preparation of affinity cosurfactant cholesteryl-D-alanyl-D-alanine.

reversed micellar phase was washed with 50 mM of phosphate buffer (pH 8) to remove the unreacted water-soluble components, and the immobilized ligand in the reverse micelles was obtained. The difference between the amount of ligand initially added into the system and that recovered in the washing solution was considered to be the amount of ligand immobilized in the reversed micelles phase.

Forward and Backward Extraction of Vancomycin

In a typical forward extraction experiment, 5 mL of reversed micelles solution composed of 0.1 M AOT with or without immobilized ligand was mixed with 5 mL of vancomycin solution (0.75 mg/mL) for 1 hour at room temperature. Backward extraction was also carried out at room temperature by mixing an equal volume of fresh aqueous solution with the reversed micelles solution for 1 hour. The pH and NaCl concentration of the aqueous solutions were varied to determine their optimal value for forward and backward extraction. Based on the amount of vancomycin in the bulk aqueous phase, the forward and backward extraction efficiency were defined as $(M_0 - M_a)/M_0$ and $M_b/(M_0 - M_a)$, respectively, where M_0 was the initial amount of vancomycin, M_a was the remaining amount after forward extraction, and M_b was the amount extracted into the backward extraction solution. The distribution coefficient of the target vancomycin in the extraction system is defined as concentration of the vancomycin in the reversed micellar phase divided by the concentration in the bulk aqueous phase. The amount of water present in the reversed micellar phase is calculated based on the molar ratio of water to the AOT surfactant, W_0, at various NaCl concentrations as reported by Fadnavis et al. (14).

Analytical Methods

For the determination of vancomycin concentration in the bulk aqueous phase after forward or backward extraction, a sample was properly diluted and its UV absorbance at 280 nm was measured in a UV/V is spectrophotometer (Shimadzu model 160 A, Japan). An identically diluted sample taken from the bulk aqueous phase of the reversed micellar extraction system without adding vancomycin was used as a blank for vancomycin determination. When the reversed micelle was applied to extract vancomycin from fermentation broth, the vancomycin concentration was determined by a HPLC system (Jasco, Japan). A reverse-phase C-18 column of 150 mm × 46 mm was employed. The mobile phase was composed of 75% 50 mM, pH 7.0 phosphate buffer, and 25% acetonitrile. The flow rate of the mobile phase was 1.0 mL/min. The ligand D-ala-D-ala concentration was determined by the Satake method (22) in which the picrylsulfonic acid reacts with the free amino group of the ligand to form an adduct that adsorbs strongly at 420 nm.

RESULTS AND DISCUSSION

Reversed Micellar Extraction

Vancomycin is a zwitterion and has an isoelectric point of 8.1 (23). At a pH lower than 8.1, the vancomycin molecule carries a net positive charge. The net positive charge on the vancomycin molecule increases as the pH decreases. On the other hand, the net negative charge of the AOT molecule is quite insensitive to pH variation because AOT is a strong anion surfactant which carries SO_3^- as its hydrophilic group. Therefore, vancomycin extraction using AOT reversed micelles will be strongly influenced by pH. As shown in Fig. 2, the forward extraction efficiency as well as the distribution coefficient decrease with an increase of pH. The extraction efficiency is about 50% at pH 3.0 and decreases to 10% at pH 8.1. On the contrary, the backward extraction efficiency increases with pH from 78% at pH 8.1 to 95% at pH 11.0. The distribution coefficient measured at backward extraction decreases with pH. This is because vancomycin becomes negatively charged at a pH higher than 8.1. As the pH increases (pH > 8.1), vancomycin becomes more negatively charged. The increased electrostatic repulsion between vancomycin and AOT molecules in reversed micelles will reduce its partitioning in the reversed

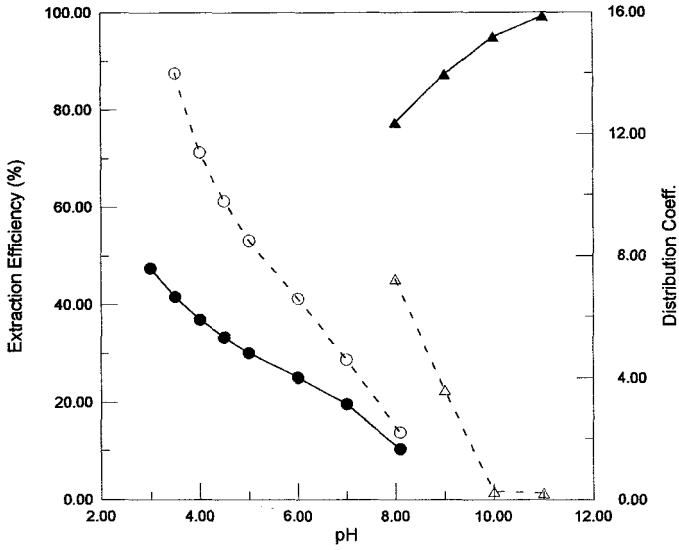

FIG. 2 The effect of pH on extraction efficiency (filled symbols) and distribution coefficient (open symbols) of vancomycin; forward (●, ○) and backward (▲, △) extraction performed in 0.2 and 0.5 M NaCl solutions, respectively.

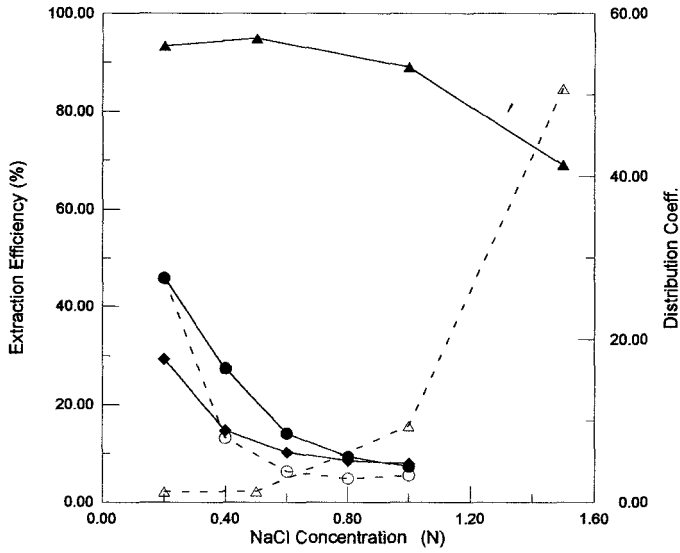

FIG. 3 The effect of NaCl concentration on extraction efficiency (filled symbols) and distribution coefficient (open symbols) of vancomycin: (●, ○) forward extraction at pH 3.5; (♦) forward extraction at pH 6.0; (▲, △) backward extraction at pH 11.0.

micellar phase and enhance its backward extraction efficiency. The difference of the distribution coefficient between forward and backward extraction at pH 8.1 is due to the different NaCl concentrations employed in the extraction systems. As shown in Fig. 3, both the forward and backward extraction efficiencies decrease with NaCl concentration. However, the distribution coefficient of backward extraction increases with NaCl concentration. The decrease of forward extraction efficiency is obviously due to the screening effect of the increased salt concentration on the electrostatic interactions between AOT molecules and vancomycin. Hydrophobic interaction is usually enhanced in a high salt concentration environment. The enhanced hydrophobic interaction between vancomycin molecules and the reversed micelle is probably the cause of the distribution coefficient increase and the extraction efficiency decrease at a high NaCl concentration during backward extraction. NaCl-induced hydrophobic interactions between vancomycin and PEG were also reported in a study of vancomycin partitioning in aqueous two-phase systems (8).

The backward extraction efficiency, as shown in Figs. 2 and 3, is much higher than that of forward extraction. This is because the calculation of forward extraction efficiency is based on the amount of vancomycin in the feed aqueous phase. The reversed micelles solution has a limited extraction capacity. Once the amount of vancomycin in the feed aqueous phase exceeds the ex-

traction capacity, the forward extraction efficiency will be lowered. On the other hand, the backward extraction efficiency is calculated based on the amount of vancomycin extracted into the reversed micelles. As long as a large amount of fresh aqueous solution is employed to backextract vancomycin, a high backward extraction efficiency can be obtained. In order to increase the forward extraction capacity, interactions other than electrostatic interactions between reversed micelles and vancomycin have to be pursued.

Ligand Immobilization to Reversed Micelles

If an affinity ligand is immobilized in the reversed micelles, additional affinity interactions between vancomycin and reversed micelles should increase the forward extraction efficiency. The immobilization of ligand D-ala-D-ala to the reversed micelles was studied by using a fixed amount of the activated cosurfactant cholesteryl-chloroformate (40 mg, ca. 88 μmol) and various amount of D-ala-D-ala (1–15 mg, ca. 7–104 μmol). Because cholesteryl-chloroformate is sparingly soluble in water, the affinity cosurfactant was prepared in situ in the presence of the AOT reversed micellar system in order to have the water-soluble ligands covalent attached to the cholesterol molecules. As shown in Fig. 4, the amount of ligand immobilized increases with the amount of ligand applied. On the other hand, the immobilization

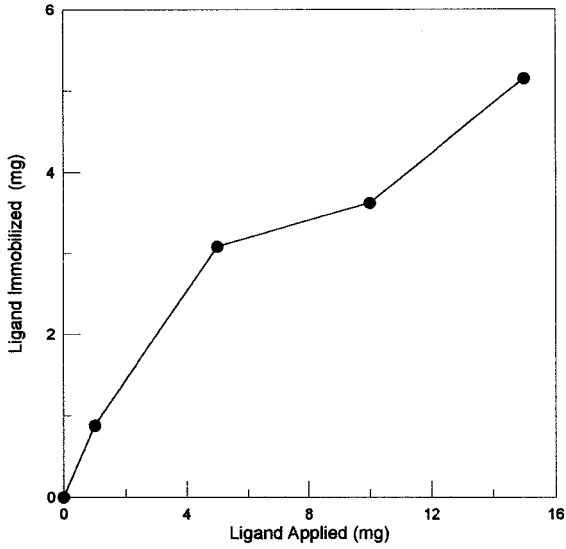

FIG. 4 The effect of the amount of added ligand on the amount of ligand immobilized to the reversed micelles.

yield, which is defined as the percentage of applied ligand immobilized, decreases as the amount of applied ligand increases. The immobilization yield decreases from 88 to 34% as the amount of applied ligand increases from 1 to 15 mg. When the amount of activated cosurfactant employed in the system is much in excess of the applied ligand, the ligand immobilization yield should be 100% if both the ligand and activated cosurfactant are in one homogeneous phase. In fact, the activated cosurfactant is only dissolved in isooctane and the ligand exists only in the aqueous core of the reverse micelle. Only cosurfactant molecules with a reactive end which contact the aqueous core of a reverse micelle can immobilize the ligand. Therefore, the limited reactive sites along the interfacial of reverse micelles will eventually be saturated with the ligand as the amount applied increases, and the result will be a decrease of immobilization yield. In order to increase the affinity extraction capacity of a reversed micelle system, the immobilization yield is usually sacrificed to have a higher amount of ligand immobilized by using an excess amount of ligand.

Affinity Reversed Micellar Extraction

Affinity reversed micellar extraction of vancomycin was carried out using systems containing various amount of the ligand D-ala-D-ala at pH 4.0 for forward extraction and pH 11.0 for backward extraction. As shown in Fig. 5, the

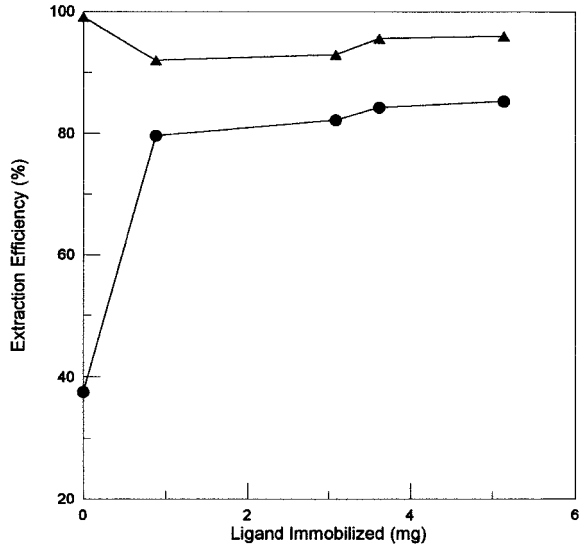

FIG. 5 The effect of the amount of immobilized ligand on the extraction efficiency of the affinity reversed micellar extraction. (●) Forward extraction at 0.2 M NaCl, pH 4.0; (▲) backward extraction at 0.5 M NaCl, pH 11.0.

TABLE 1
Effect of Ligand Type on Affinity Extraction Efficiency and Distribution Coefficient of Vancomycin[a]

Ligand	No	L-ala	DL-ala-DL-ala	D-ala-D-ala
Immobilization yield (%)	—	36.8	38.4	40.3
Forward extraction:				
Extraction efficiency (%)	37.5	39.4	87.7	89.5
Distribution coefficient	11.7	12.7	140.4	166.7
Backward extraction:				
Extraction efficiency (%)	99.1	98.2	84.4	92.6
Distribution coefficient	0.2	0.3	3.3	1.4
Total recovery (%)	37.2	38.7	74.0	82.9

[a] Feed aqueous solution containing 0.75 mg/mL vancomycin in buffer (pH 4.0, 0.2 N NaCl), backward extraction solution pH 11.0, 0.5 N NaCl; 15 mg ligand was applied to be immobilized in reversed micelles.

forward extraction efficiency increases significantly, from 37 to 80%, when an affinity ligand is employed. However the amount of immobilized ligand does not affect the forward extraction efficiency much. The extraction efficiency increases from 80 to 84% when the immobilized ligand content increases from 0.88 to 5 mg. This is because the amount of applied immobilized ligand D-ala-D-ala (0.88 mg, ca. 6.2 μmol) exceeds the amount of vancomycin added to the system (2.52 μmol). Since most of vancomycin molecules are extracted into reversed micelles either via electrostatic or affinity interactions, a further increase of immobilized ligand does not help to enhance the forward extraction much. The backward extraction efficiency, on the other hand, is not affected by the presence of immobilized affinity ligand and remains at about 95%. In other words, the backward extraction conditions (0.5 M NaCl, pH 11.0) are also very effective for backextracting vancomycin from the affinity reversed micelles. Based on total vancomycin recovery, the best extraction yield (3.0 mg vancomycin per mg of immobilized ligand) is obtained in an extraction system using 0.88 mg immobilized ligand.

In addition to D-ala-D-ala, DL-ala-DL-ala and L-ala were also employed as affinity ligands to study the affinity reversed micellar extraction of vancomycin. The forward and backward extractions were carried at conditions of 0.2 M NaCl, pH 4 and 0.5 M NaCl, pH 11.0, respectively. As shown in Table 1, the different ligands do not affect their immobilization yield to the reversed micelles much. However, they do affect the forward extraction efficiency significantly. As expected, L-ala as a ligand does not increase the forward extraction efficiency and distribution coefficient appreciably. DL-ala-DL-ala and D-ala-D-ala, on the other hand, both increase the

forward extraction efficiency and distribution coefficient significantly, from about 40 to 90% and from 11.7 to 166.7, respectively. This indicates that DL-ala-DL-ala also contributes its affinity interactions with vancomycin to increase the forward extraction efficiency. D-ala-D-ala is generally known as the affinity ligand for vancomycin for many years, but the utilization of racemic dipeptide DL-ala-DL-ala as an affinity ligand for vancomycin has not been reported yet. The racemic dipeptide DL-ala-DL-ala should consist of four different dipeptides, namely D-ala-D-ala, D-ala-L-ala, L-ala-L-ala, and L-ala-D-ala. According to the study of the combination of vancomycin with peptides carried out by Neito and Perkins (24), only D-ala-D-ala in the DL-ala-DL-ala mixture will combine with vancomycin. In comparison with D-ala-D-ala, using DL-ala-DL-ala as the affinity ligand should result in a much lower forward extraction efficiency because only 25% of the DL-ala-DL-ala molecules are effective affinity ligands. However, as shown in Table 1, the forward extraction efficiency of using DL-ala-DL-ala as the ligand is only slightly lower than that of using D-ala-D-ala. The reason for this result is because the amount of effective ligand D-ala-D-ala (9 μmol) in the total DL-ala-DL-ala employed (36 μmol) is still in great excess of the amount of vancomycin (2.52 μmol) loaded in the system.

Affinity Extraction Vancomycin from Fermentation Broth

Vancomycin can be produced by *Streptomyces orientalies* (ATCC19795) according to the cultivation conditions reported by McIntyre et al. (25). When this microorganism was cultivated in this study, however, a very low concentration of vancomycin was detected in the fermentation broth. Since a higher vancomycin concentration will make it easier for affinity reversed micellar extraction to demonstrate its extraction capability, pure vancomycin was added to the fermentation broth. After adjusting the pH to 3.0, the broth was extracted with affinity reversed micelles using D-ala-D-ala as a ligand. Figure 6 shows HPLC analysis of samples taken from each extraction steps. As shown in the HPLC chromatogram of the fermentation broth before extraction (Fig. 6a), vancomycin appears at 18.3 minutes along with a large amount of unwanted impurities. The vancomycin peak decreases significantly after affinity reversed micellar extraction, but the peaks of unwanted impurities do not show any appreciable change (Fig. 6b). Evidently vancomycin can be specifically extracted into the reversed micelles with a very high efficiency, even in the fermentation broth. When a 0.5 M NaCl solution of pH 11.0 is used as the backward extraction solution, vancomycin can be recovered along with a small amount of unwanted impurities (Fig. 6c) but its purity is improved to a great extent. Based on the peak area of vancomycin shown in Figs. 6(a) and 6(c), the total recovery of vancomycin was about 40% which is much less than that obtained in studies using pure vancomycin

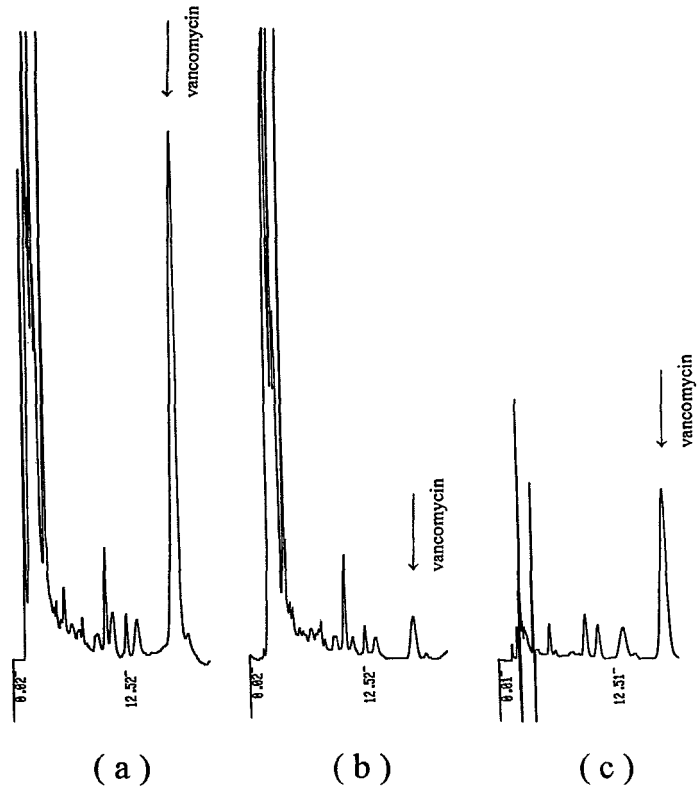

FIG. 6 HPLC chromatograms during affinity reversed micellar extraction of vancomycin from fermentation broth. (a) Fermentation broth before extraction; (b) fermentation broth after extraction; (c) bulk aqueous solution after backward extraction.

(Table 1). The extraction yield is about 1.2 mg vancomycin per mg immobilized ligand. The low recovery yield is probably caused by the insoluble particles and surface-active agents produced during fermentation. These contaminants may affect the formation of reversed micelles by AOT and consequently reduce the vancomycin extraction efficiency. Fermentation broth pretreated by ultrafiltration may increase the recovery yield of affinity reversed micellar extraction.

CONCLUSION

Vancomycin is able to be extracted into AOT-based reversed micelles via electrostatic interactions. In the range of pH less than the pI of vancomycin

(8.1), both a lower pH and salt concentration of the feed solution enhance the forward extraction efficiency. The backward extraction efficiency, on the other hand, increases with pH when the pH of the solution is higher than the pI of vancomycin. In addition to the electrostatic interactions, affinity interactions resulting from the immobilized affinity ligand can increase the extraction capacity and specificity. The existence of the affinity cosurfactant cholesteryl D-ala-D-ala in a reversed micellar extraction system makes the forward extraction efficiency increase twofold. Besides D-ala-D-ala, the racemic DL-ala-DL-ala can also act as an affinity ligand for vancomycin extraction. HPLC analysis demonstrates that affinity reversed micelles can extract vancomycin from fermentation broth with very high specificity. The purity of recovered vancomycin improves significantly after backward extraction. Although affinity reversed micellar extraction can not obtain 100% pure vancomycin, its easy scale-up ability make it very suitable for use as the first isolation step in a large-scale process of vancomycin recovery. The vancomycin recovered from this step can be applied directly to chromatography to obtaining 100% pure vancomycin.

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