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Nonionic Surfactant-Mediated Affinity Cloud-Point Extraction of Vancomycin

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ABSTRACT

The partitioning behaviors of vancomycin in a temperature-induced nonionic surfactant two-phase system were studied. *N*-Decyltetra(ethylene oxide) ($C_{10}E_4$) was employed as the nonionic surfactant. Vancomycin had a preference for the micelle-rich top phase under most experimental conditions. However, at pH 4 vancomycin preferred to stay in the micelle-poor bottom phase. An affinity cosurfactant, D-alanyl-D-alanine modified cholesterol, was employed in the extraction system to increase the partition coefficient of vancomycin. The partition coefficient increased significantly from 0.87 to 15.98 when an affinity cosurfactant was employed. Vancomycin could form a micelle, and its critical micelle concentration (CMC) was determined by the methyl orange binding technique and the surface tension method. The micelle formation of vancomycin was considered to induce the lower partition coefficient and a significant increase of the partition coefficient in the presence of the affinity cosurfactant at pH 4. Direct recovery of vancomycin from fermentation broth using affinity extraction was also demonstrated.

Key Words. Cloud-point extraction; Temperature-induced phase separation; $C_{10}E_4$; Vancomycin; CMC of vancomycin

INTRODUCTION

Glycopeptide antibiotic vancomycin is a clinically important therapeutic agent used for the treatment of infection owing to methicillin-resistant *Staphy-*

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lococcus aureus (MRSA) (1). In additional to its medical application, vancomycin is one of the more popular and successful chiral selectors used in separation science for the HPLC, TLC, and CE separation of optical isomers (2). It is produced by fermentation of the actinomycete strain *Amycolatopsis orientalis* [formerly classified as *Nocardia* and *Streptomyces orientalis* (3)]. The recovery of vancomycin from fermentation broth is a challenging task because the broth contains a large amount of metabolites, proteins, salts, etc. along with dilute vancomycin [ca. 80 mg/L (3)]. Industrially, its recovery process usually consists of several separation unit operations such as filtration, solvent extraction, ion-exchange adsorption, and precipitation (4). Since antibiotics are rather unstable molecules, it is desirable that product recovery is done under very mild conditions by a quick and efficient method.

Vancomycin was found to bind very tightly and specifically to the dipeptide D-alanyl-D-alanine at the carboxyl terminus with a dissociation constant of about 10^{-7} M (5). Based on this fact, the recovery of vancomycin and several other glycopeptide antibiotics of the vancomycin family using D-alanyl-D-alanine as the affinity ligand have been carried out by chromatography (6, 7), aqueous polymer–polymer two-phase extraction (8, 9), ultrafiltration (10, 11), and reverse micellar extraction (12). In comparison with other separation operations, extraction is the most feasible industrial process because of its ease of scale-up. Instead of using an organic solvent or a water-soluble polymer, a nonionic surfactant can also be employed to form two liquid phases for extraction. Aqueous solutions of many nonionic surfactants become turbid on heating to a temperature known as the cloud point, following which there is a separation of the solution into micelle-rich and the micelle-poor phases. The advantages of using cloud-point extraction is that only a small amount of nonionic surfactant is required to generate the two phases and an affinity cosurfactant can be incorporated into the system to increase the extraction selectivity. A schematic diagram of the nonionic surfactant-mediated affinity two-phase extraction system is shown in Fig. 1. In this paper we present our preliminary feasibility studies on vancomycin recovery by affinity cloud-point extraction. The affinity cosurfactant was prepared by coupling the ligand D-alanyl-D-alanine to the chlesterol molecule. The nonionic surfactant *N*-decyltetra(ethylene oxide) ($C_{10}E_4$) along with the affinity cosurfactant were used to extract vancomycin from the fermentation broth. With the extraction temperature higher than the cloud point of $C_{10}E_4$ (19°C), vancomycin partitioning between the micelle-rich top phase and micelle-poor bottom phase was studied. The effect of pH and ligand on vancomycin extraction efficiency and partitioning behaviors were also investigated.

MATERIALS AND METHODS

N-Decyltetra(ethylene oxide) ($C_{10}E_4$) was purchased from Nikkol Chemical Co. (Kyoto, Japan). Vancomycin hydrochloride, D-alanyl-D-alanine, L-ala-

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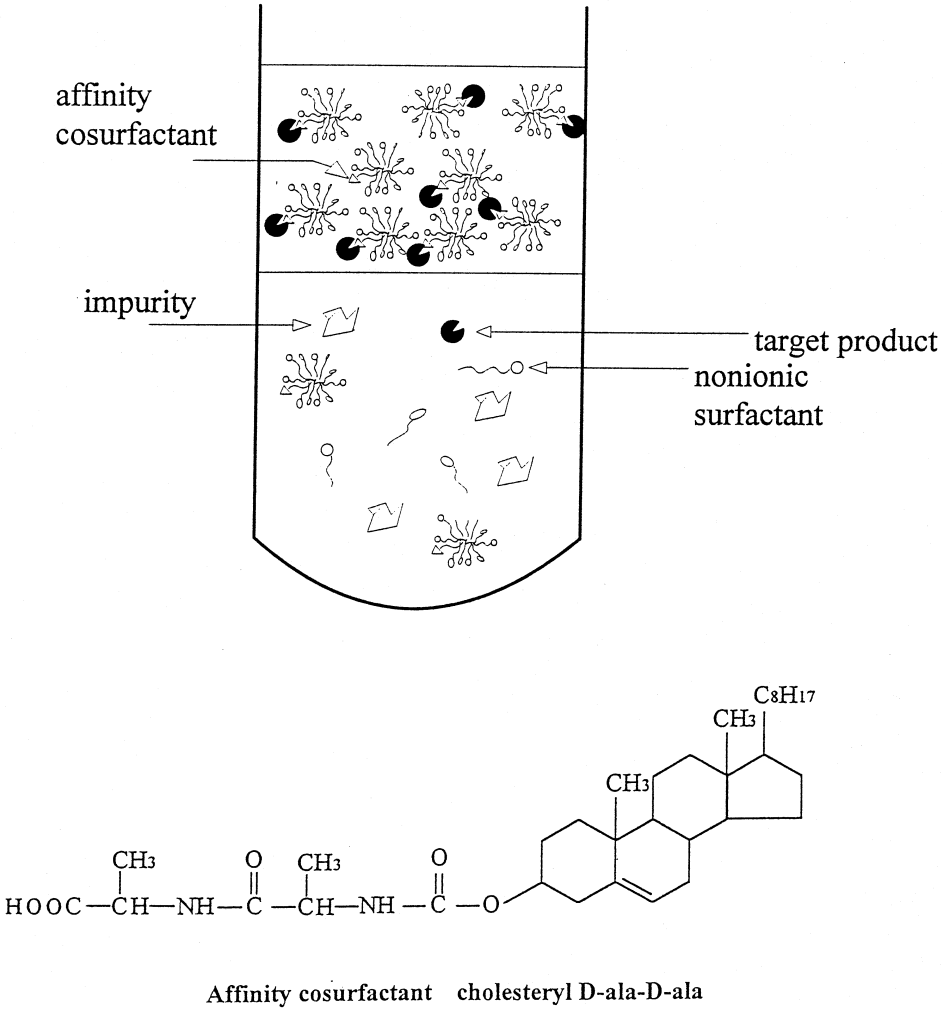

Affinity cosurfactant cholesteryl D-ala-D-ala

FIG. 1 Schematic diagram of nonionic surfactant-mediated affinity cloud-point extraction; the affinity cosurfactant was prepared by coupling the affinity ligand D-ala-D-ala to cholesterol.

nine, and cholesteryl chloroformate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Affinity cosurfactant preparation

Affinity ligand D-alanyl-D-alanine (15 mg) was dissolved in 0.5 mL pH 10 borate buffer. Nonionic surfactant $C_{10}E_4$ (0.5 g) and cholesteryl chloroformate (40 mg) were added to the ligand solution and mixed for 20 hours at room temperature. Distilled water (5 mL) was then mixed with the reaction solution to extract and remove the uncoupled ligand. After phase separation, the micelle-rich top phase, which contained the most of affinity cosurfactant, was saved as the affinity extraction solvent.

Vancomycin Extraction

Vancomycin extraction without an affinity cosurfactant was performed by mixing 5 mL vancomycin solution (0.94 mg/mL) with 0.5 g $C_{10}E_4$ at 25°C. For affinity extraction, the affinity extraction solvent (1.4 mL) was mixed with 5 mL of vancomycin solution (0.94 mg/mL). Two clear liquid phases were obtained after the extraction mixture settled for 6 hours at 25°C. An aliquot of sample was taken from bottom phase for vancomycin concentration determination by HPLC analysis. The volume of each phase was measured by using a 10-mL graduated cylinder. The vancomycin concentration in the top phase was calculated by mass balance. The extraction efficiency was defined as the percentage of the initial amount of vancomycin in the feed solution that was extracted into the micelle-rich top phase. The partition coefficient was defined as the ratio of vancomycin concentration in the top phase to that in the bottom phase.

Determination of the Critical Micelle Concentration (CMC) of Vancomycin

Determination of the CMC of vancomycin by the dye-binding technique followed the method described by Corti et al. (13). Methyl orange (0.04 mM) was prepared in 50 mM pH 4 citrate/phosphate buffer and pH 7 phosphate buffer. A vancomycin solution prepared in the corresponding buffers was mixed with the methyl orange solution and the absorbance of the solution was measured at 484 nm. CMC is defined as the concentration at which a sharp decrease in A_{484} is observed. A surface tension method was also employed to study the micelle formation of vancomycin. Surface tensions of vancomycin solutions were measured using a video digital pendant bubble tension meter build by Dr. Lin's surfacant laboratory of NTUST.

Analytical Methods

Vancomycin concentration after proper dilution was determined by using HPLC (Jasco HPLC System, Japan). A reversed-phase C-18 column of 150 mm $\times$ 46 mm was employed. The mobile phase consisted of 88% 50 mM pH 8.0 phosphate buffer and 12% acetonitrile. The flow rate of the mobile phase was 1.0 mL/min and the column eluent was detected at 210 nm.

RESULTS AND DISCUSSION

Cloud-Point Extraction

The temperature versus surfactant concentration phase diagram of $C_{10}E_4$ system has been reported by Liu et al. (14). At temperatures higher than its

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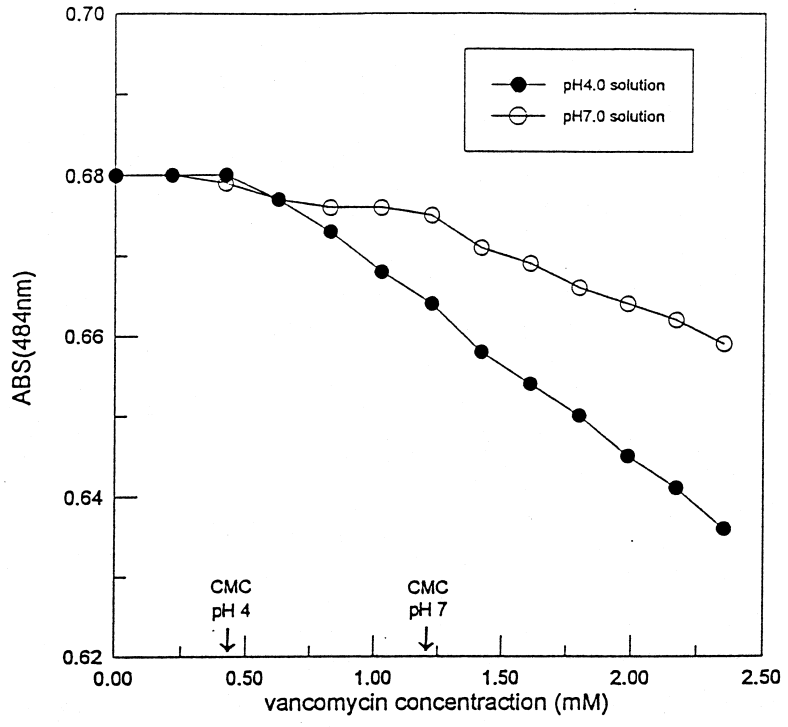
cloud point (ca. 19°C), an aqueous solution containing the proper $C_{10}E_4$ concentration will form a micelle-rich top phase and a micelle-poor bottom phase. Table 1 shows the effect of pH and Na_2SO_4 on the phase volume ratio, partition coefficient, and extraction efficiency of vancomycin in the $C_{10}E_4$ two-phase system. Since vancomycin easily degrades in an alkaline environment (15), only the acidic pH effect was studied. In comparison with the effect of Na_2SO_4, pH does not affect the phase volume ratio much. Only at pH 2 does the phase volume ratio increase appreciably. The phase volume ratio decreases to half in the presence of 0.25 M Na_2SO_4. Na_2SO_4 is known as a salting-out agent which can increase the hydrophobic interaction of solutes. A possible mechanism for the volume decrease of the top phase is that Na_2SO_4 enhances the hydrophobic interaction between the hydrophobic groups of $C_{10}E_4$. The enhanced interaction reduces the size of the micelles; as a consequence, the volume of the micelle-rich top phase is reduced. The vancomycin extraction efficiency also decreases in the presence of Na_2SO_4 because the volume ratio decreases. Since the top phase becomes proportionally smaller than the bottom phase, less vancomycin is recovered in the top phase. Vancomycin prefers to stay in the micelle-rich top phase through its interactions with $C_{10}E_4$ as indicated by its partition coefficient which is higher than unity at pH 2 and 7. However, its partition coefficient is lower than unity at pH 4. This anomalous partitioning behavior is probably due to the formation of micellar aggregates of vancomycin at pH 4. One vancomycin group antibiotics, teicoplanin, has been shown to form micellar aggregates due to its amphiphilic structure (13). Its critical micelle concentration (CMC) is 0.2 mM at pH 7.4 and 0.8 mM at pH 8.0. Vancomycin may also form micellar aggregate as teicoplanin. As shown in Fig. 2(a), the CMC of vancomycin is about 0.4 mM at pH 4 and 1.2 mM at pH 7 as determined by the dye-binding method. A surface

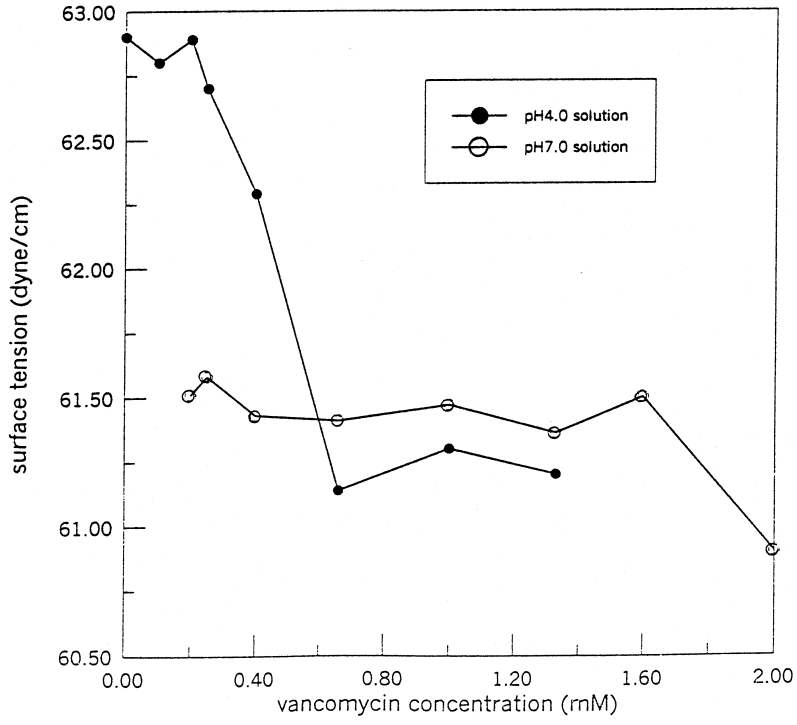
TABLE 1
pH and Na_2SO_4 Effect on Vancomycin Partitioning in the $C_{10}E_4$ Two-Phase System[a]

	pH					
	2		4		7	
Na_2SO_4 (M)	0	0.25	0	0.25	0	0.25
Extraction efficiency (%)	51.24	36.08	32.47	17.83	41.77	24.76
Partition coefficient (C_T/C_B)	1.43	1.87	0.87	0.77	1.39	1.21
Volume ratio (V_T/V_B)	0.73	0.3	0.55	0.28	0.51	0.27

[a] The two phases were formed by mixing 0.5 g $C_{10}E_4$ with 5 mL vancomycin solution (0.94 mg/mL).



(a)



(b)

FIG. 2 Determination of CMC of vancomycin using (a) methyl orange binding technique and (b) surface tension method.

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tension method was also employed to study the micelle formation of vancomycin. As shown in Fig. 2(b), the surface tension starts to decrease appreciably at 0.2 mM for a vancomycin solution of pH 4. On the other hand, surface tension shows an appreciable decrease at 1.6 mM for a solution of pH 7. This again shows that the CMC of vancomycin at pH 7 is higher than that at pH 4. Since the vancomycin concentration used in this study is about 0.6 mM (higher than the CMC determined by both methods at pH 4, but lower than at pH 7), vancomycin probably will form micelles at pH 4 rather than at pH 7. In addition, at a much lower pH such as pH 2, the amphiphilic property of vancomycin may change dramatically and reduces its tendency for micellization because the CMC could not be determined by either dye-binding or surface tension methods. Therefore, at the pH values studied, vancomycin will form micelles only at pH 4. We speculate that the excluded-volume interaction (14) between vancomycin micelles and $C_{10}E_4$ micelles in the micelle-rich phase reduces the partition coefficient of vancomycin. In other words, the vancomycin micelles are pushed into the phase which has a larger available free volume (in this case the micelle-poor bottom phase).

Affinity Cloud-Point Extraction

In order to increase the partition coefficient of vancomycin in the temperature-induced nonionic surfactant two-phase system, an affinity cosurfactant made of cholesterol and an affinity ligand D-ala-D-ala was prepared and employed. Cholesterol is a hydrophobic molecule and has a very low solubility in aqueous solution. $C_{10}E_4$ was employed to enhance its solubility during affinity cosurfactant preparation. By measuring the UV absorbance of both phases at 270 nm for cholesterol concentration determination, the partition coefficient of affinity cosurfactant was determined to be about 277. In other words, most of the prepared affinity cosurfactant cholesteryl-D-ala-D-ala stays in the micelle-rich top phase. Table 2 shows the effect of cholesteryl-D-ala-D-ala on vancomycin partitioning. The partition coefficient increases 16-fold from 0.87 to 15.98 when cholesteryl-D-ala-D-ala is employed in the two-phase

TABLE 2
Effect of Affinity Cosurfactant on Vancomycin Partitioning at pH 4 in the $C_{10}E_4$ Two-Phase System[a]

	Control	L-ala	D-ala-D-ala
Extraction efficiency (%)	19.4	20.4	81.7
Partition coefficient (C_T/C_B)	0.87	0.91	15.98

[a] The two phases were formed by mixing 1.4 mL affinity extraction solvent with 5 mL vancomycin solution.

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TABLE 3
pH Effect on Vancomycin Partitioning in Affinity Two-Phase System Using Cholesteryl-D-ala-D-ala as the Affinity Cosurfactant[a]

	pH		
	2	4	7
Extraction efficiency (%)	59.65	81.74	74.39
Partition coefficient (C_T/C_B)	5.28	15.98	10.37

[a] The two phases were formed by mixing 1.4 mL affinity extraction solvent with 5 mLvancomycin solution.

system. When L-ala is used as the ligand, the extraction efficiency as well as the partition coefficient have no appreciable increase. Clearly, D-ala-D-ala is an effective affinity ligand for vancomycin. In comparison with extraction without an affinity cosurfactant, the partition coefficient of vancomycin increases at least 3-fold in the affinity extraction system, as shown in Tables 1 and 3. The increase of the partition coefficient is most notable at pH 4 (0.87 for plain extraction and 15.98 for affinity extraction). The anomalously high partition coefficient at pH 4 may be attributed to the micelle formation of vancomycin at pH 4, although the self-micellization of vancomycin will reduce its partitioning into the micelle-rich top phase due to the excluded-volume effect mentioned earlier. However, Rao et al. (16) recently showed that synthesized trivalent vancomycin binds a trivalent ligand derived from D-ala-D-ala with an extremely high affinity (dissociation constant $K_d \approx 10^{-17}$ M). The affinity binding of the trivalent system is much higher than that of the monovalent system ($K_d \approx 10^{-7}$ M). Therefore, the vancomycin micelle may be considered to be a multivalent vancomycin, and the affinity cosurfactant-incorporated $C_{10}E_4$ micelle may act as a multivalent ligand. The affinity between the multivalent vancomycin and multivalent ligand should be much higher than in the monovalent vancomycin system. Corti et al. (13) reported that the binding capacity of an affinity resin with D-ala-D-ala ligand for the micelle form of teicoplanin is 3.6 time higher than for the monomeric form of teicoplanin. Based upon these facts, we speculate that the effect of enhanced affinity interaction exceeds the excluded-volume effect (both effects result from vancomycin self-micellization) to such a great extent that the partition coefficient of vancomycin increases significantly at pH 4.

An affinity solvent consisting of $C_{10}E_4$ and the affinity cosurfactant cholesteryl-D-ala-D-ala was also employed to extract vancomycin directly from fermentation broth. The *Streptomyces orientalis* (ATCC19795) strain was cultivated using the same medium and conditions as described by McIntyre et al. (3) for vancomycin production. However, a very low titer of van-

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comycin was detected in the fermentation broth. Pure vancomycin was then added to the fermentation broth for the study of affinity extraction. The pH of the fermentation broth was adjusted to 4 using hydrochloric acid. The affinity solvent (1.4 mL) was mixed with 5 mL of fermentation broth to extract vancomycin. After phase separation, the bottom phase was replaced with 5 mL of stripping solution (0.5 M NaCl in 50 mM, pH 11 phosphate/NaOH buffer). Figure 3 shows the HPLC analysis of vancomycin in the fermentation broth before and after extraction, and in the stripping solution after backward extraction. After affinity extraction, the peak of vancomycin in the HPLC chromatogram shows a significant decrease, which indicates that vancomycin is extracted into the top phase. Vancomycin in the micelle-rich top phase can be backward extracted into the stripping solution but not completely. By comparing the peak area of vancomycin shown in Figs. 3(a) and 3(c), the vancomycin recovery yield is about 37%. The recovery yield can be improved further by optimizing the affinity extraction and stripping conditions.

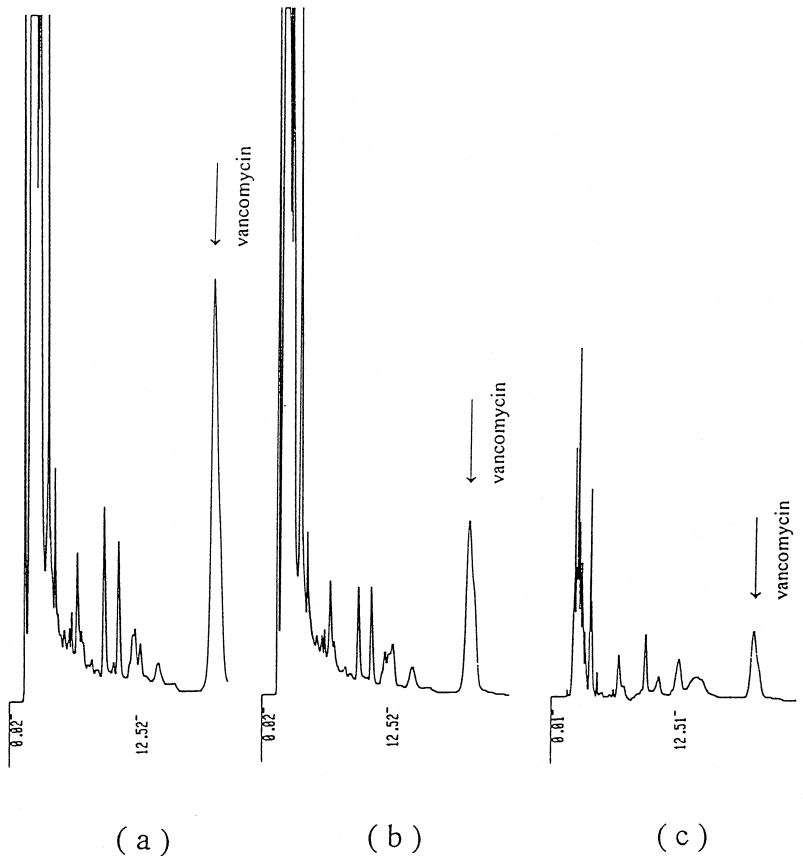

FIG. 3 HPLC chromatograms of affinity cloud-point extraction of vancomycin from fermentation broth. (a) Fermentation broth before extraction; (b) fermentation broth after extraction; (c) stripping solution after backward extraction.

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CONCLUSION

Vancomycin shows an anomalous partitioning behavior in the $C_{10}E_4$ mediated two-phase system. Its partition coefficient is greater than unity at pH 2 and 7 but less than unity at pH 4. A possible mechanism which explains the anomalous partitioning behavior is based on the micelle formation of vancomycin at pH 4. Vancomycin of the concentration used in this study could form micelles only at pH 4 as demonstrated by its CMC as measured by dye-binding and surface tension methods. Vancomycin micelles partition favorably into the micelle-poor bottom phase due to the excluded-volume effect. The partition coefficient of vancomycin at pH 4 increases 16-fold when an affinity cosurfactant is employed. The significant increase of the partition coefficient at pH 4 during affinity extraction may also be due to the self-micellization of vancomycin. Vancomycin micelles may induce a stronger affinity binding than monomeric vancomycin toward the affinity cosurfactant. The effect of enhanced affinity binding exceeds the effect of excluded-volume to such a large great extent that the result is a significant increase of vancomycin partitioning into the affinity cosurfactant-incorporated micelle-rich top phase.

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