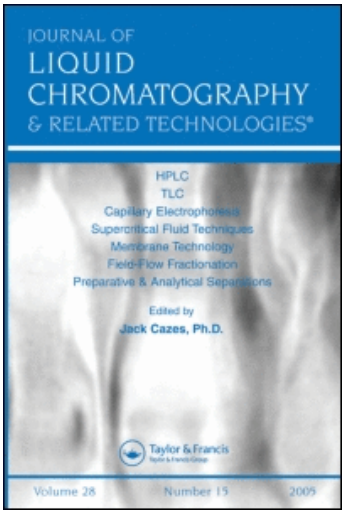

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APPLICATION OF PREPARATIVE CHROMATOGRAPHY TO THE ISOLATION OF EPIRUBICIN FROM RAW PRODUCT

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APPLICATION OF PREPARATIVE CHROMATOGRAPHY TO THE ISOLATION OF EPIRUBICIN FROM RAW PRODUCT

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ABSTRACT

Epirubicin (4'-epidoxorubicin) has been found to be useful in the treatment of cancer. The isolation of milligram amounts of epirubicin from raw product was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) on Kromasil KR100-10C_{18} (250 × 4.6 mm I.D., 10 μm) column. The purity of epirubicin is required to be greater than 99%. First, is adjusting the mobile phase composition, the column temperature, and the flow-rate to increase the separation factor between the desired component and the nearest neighbor. Then, it is possible to increase the column efficiency and to reduce the time of separation required. Besides, displacement effects could be utilized efficiently to increase the sample size and, therefore, the production rate. The location of the cut points for the collection of target compound was determined by use of detector response levels.

This method can be used as a guide to scale up the industrially preparative separation of epirubicin.

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INTRODUCTION

High-performance preparative liquid chromatography (HPPLC), because of its higher column efficiencies, has been gaining popularity over the past few years. It permits more difficult separations to be conducted efficiently and quickly. This feature is of importance, especially in the pharmaceutical industry today, since new products have to be introduced to the market as quickly as possible.

Epirubicin (4'-epidoxorubicin), an anthracycline antitumour antibiotic, is an analog of doxorubicin modified in the sugar moiety and in which the stereochemistry, at the hydroxyl group bearing C-4', has been inverted (Fig. 1). An updated review of its pharmacodynamics study has shown that epirubicin may be administered alone, or in combination with other agents, both to patients with superficial bladder cancer and to those with metastatic diseases.(1) The drug has also established activity in the treatment of breast cancer, liver cancer, ovarian cancer, and gastrointestinal tract cancer. However, the purification of epirubicin by HPPLC has seldom been found in the literature.

The optimization of experimental conditions for preparative chromatography is of importance and a self-consistent process, which cannot be resolved in separable effects. Several optimization theories have been presented with different assumptions.(2-10) This paper illustrated a practical example of optimization for the isolation of a desired component from raw product using non-linear chromatography. First, in isolating the epirubicin from the impurities present in raw product, it is important to adjust the mobile phase composition, column temperature, and the flow-rate for an improvement of the column efficiency and the resolution between the desired component and the nearest neighbor. Then, the non-

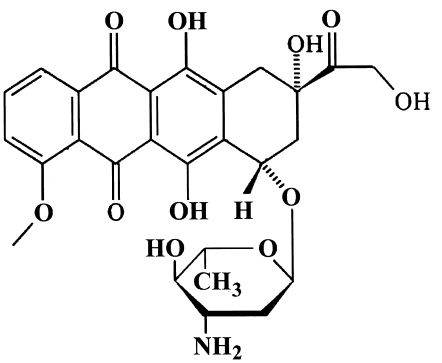

Figure 1. The molecular structure of epirubicin.

linear chromatographic behavior of epirubicin was studied by increasing the load of raw product, instead of spiked sample for adsorption isotherm measurement that is tedious, laborious, and skillful as well. The changes of the peak shape with increasing the amount of sample injected, have indicated the competitive adsorption behavior of the target compound in real sample consisting of multi-component. Besides, displacement effects could be utilized efficiently for an increase of the sample throughput and, therefore, the production rate. This method can be used as a guide to scale up the industrially preparative separation of epirubicin.

EXPERIMENTAL

Apparatus and Materials

The TSP liquid chromatograph (TSP, San Jose, CA, USA) consisted of a P4000 pump, an AS 3000 autosampler, and a Spectra FOCUS diode array detector. Chromatographic system control, data acquisition, and chromatographic analysis were exerted with TSP PC1000 Chromatography Manager software (3.0 version).

Kromasil KR100-10C_{18} 250 × 4.6 mm I.D. (10 μm) column was obtained from Eka Chemicals (Bohus, Sweden). All solvents and reagents used for method development and the purity analysis were of HPLC grade. The water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA.). Epirubicin raw product solution, epirubicin, doxorubicin, daunorubicin, and epi-daunorubicin standards were obtained as a gift from Hisun Pharmaceutical Co Ltd. (Zhejiang, China).

Experimental Procedure

Preparative separations were performed using methanol-water (50:50, v/v, adjusting pH to 2.4 with formic acid) as mobile phase. The flow-rate was 0.4 mL/min and the column temperature was 40°C. 80 μL raw product solution without dilution was injected into the column. The eluted epirubicin from the column was monitored at 280 nm and collected from the starting cut point at 400 mAU to the ending cut point at 100 mAU, according to the signal level of the detector. After the collection was completed, the column was washed with methanol at 1.0 mL/min for 5 min to remove all impurities strongly retained on the column, and then re-equilibrated with the initial mobile phase for 10 min.

Analysis of purity for the purified epirubicin was conducted at room temperature. The mobile phase was methanol-acetonitrile-water at the ratio of

28:17:55 (v/v) and pH 2.4 adjusted with formic acid. Flow-rate was 0.8 mL/min. The detection of peaks was at U.V. 254 nm.

RESULTS AND DISCUSSION

Optimization of Experimental Conditions

Optimization of preparative separation looks formidable, because of the large number of parameters that should be considered. The solution for optimization problems can be considerably simplified with the help of theory.(2) Method development under analytical conditions makes an excellent starting point. Therefore, the concentration of raw product injected into the system should be satisfactory to fall into the linear range of corresponding adsorption isotherm; then experimental parameters such as the mobile phase composition, the column temperature, and the flow-rate were optimized.

Selection of the Mobile Phase

In order to simplify the after-treatment process of chromatographic separation, it is important to develop a method using the mobile phase without salt, or with a volatile salt. The optimal mobile phase is usually achieved by maximizing the difference in retention values of the desired compound and the adjacent contaminants, i.e., improving the selectivity of the compounds concerned and, thereby, effectively augmenting the capacity of the separation.(3) Therefore, a binary mobile phase with isocratic elution was used in our study, which was preferable for preparative separation.

The diluted raw product was analyzed on a KR100-10C_{18} column using methanol-water (60:40, v/v) as mobile phase at different pH's adjusted by phosphoric acid, acetic acid, and formic acid, respectively. The results indicated:

1. Mobile phase under neutral and acidulous conditions could not separate epirubicin from most other component present in the raw product. Furthermore, the column has shown an irreversible adsorption to the sample. The peak height of the sample was gradually enhanced by continuous injections of the sample at first, and finally, got to be stable after the fourth injection (Fig. 2).

2. Most components in the raw product could be completely separated from epirubicin by the mobile phase at pH 2.4. The separated peaks of 1, 4, and 5, shown in Fig. 3, were identified by the standards individually. They are doxorubicin, daunorubicin, and epi-daunorubicin. It was found that doxorubicin was the nearest neighbor of the desired component – epirubicin, so the solution for the

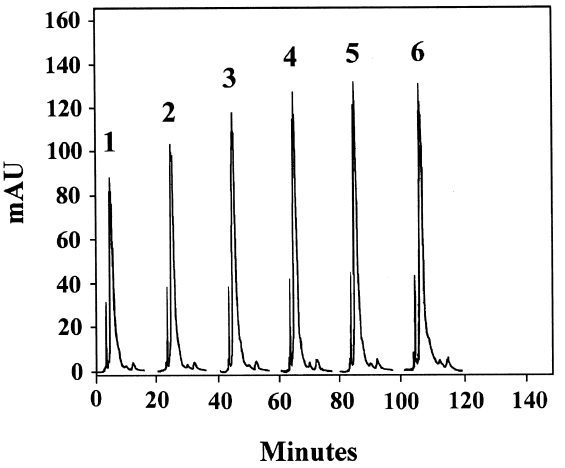

Figure 2. The irreversible adsorption of epirubicin on the column. Mobile phase: methanol-water (60:40,v/v, pH=7.0). Flow-rate: 0.8mL/min, at room temperature. Sample: 20 μL raw product solution diluted 1:10 with mobile phase.

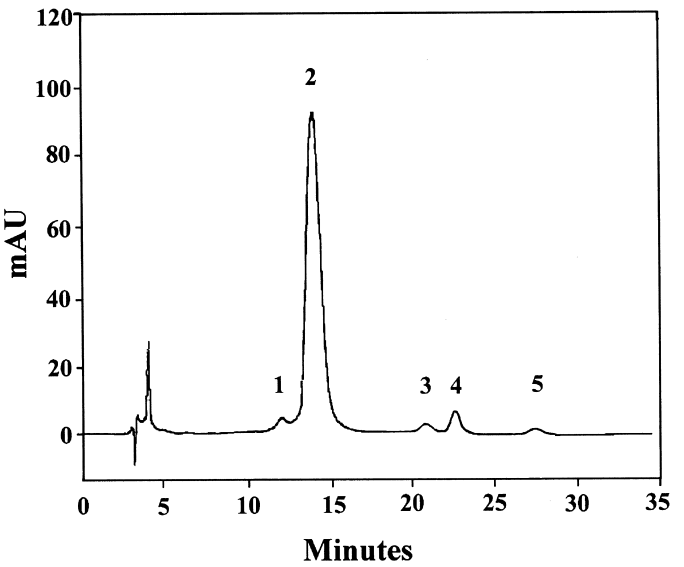

Figure 3. The chromatogram of raw product. Mobile phase: methanol-water (60:40, v/v, pH=2.4). Flow–rate: 0.8mL/min, at room temperature. Peak identification: 1=doxorubicin, 2=epirubicin, 3=unknown impurity, 4=daunorubicin, 5=epi-daunorubicin. Other conditions as given in the text.

Table 1. Summary of Chromatographic Data

Methanol-Water (v/v,pH=2.4)	Flow-Rate (mL/min)	Temperature (°C)	t_R (min)	k'*	α*	R*	Plate/ Meter
60:40	0.8	18	13.7	4.1	1.1	1.4	11280
50:50	0.8	18	27.5	8.2	1.3	2.7	24500
50:50	0.8	40	16.2	4.7	1.3	2.8	37060
50:50	0.4	40	32.8	4.6	1.3	2.9	41030
50:50	0.25	40	50.1	5.1	1.3	3.0	42020

* k' is capacity factor; α is separation factor; R is resolution.

isolation problem depended mainly on how well separated doxorubicin was from epirubicin.

With decreasing the content of methanol in the mobile phase, both the resolutions (Table 1) and the retention times of the solutes were increased dramatically. Therefore, using the mobile phase consisting of methanol-water (50:50, v/v, pH=2.4) is a reasonable compromise between separation time and resolution. (3) Adjusting pH 2.4 of mobile phase with different acids has indicated no significant differences of the separations as shown in Table 2. So, formic acid was selected due to its volatility and being easily removed in the after-treatment process.

The unknown component (peak 3 in Fig. 3) was not identified yet. However, it was found that the retention value of the corresponding peak was not affected by the pH of the mobile phase. In other words, the elution orders of all other impurities but the unknown impurity were changed with adjusting the pH of the mobile phase. The result indicates that the unknown impurity is probably a nonionic compound under the experimental conditions.

Table 2. Effects of Adjusting pH of Mobile Phase with Different Acids

The Composition of Mobile Phase	Acids	t_R (min)	$W_{1/2}$ (min)	As*
Methanol-water	Phosphoric acid	13.2	0.5	1.3
(60:40, v/v, pH=2.4)	Acetic acid	13.0	0.7	1.4
	Formic acid	13.7	0.6	1.4

* As is symmetry factor.

Selection of the Column Temperature and the Flow-Rate

It is often said that efficiency is not essential in preparative separation, because the load on the column is usually so large that it destroys the column efficiency. This statement is incorrect. In order to increase the production rate of each cycle, higher column efficiency is the critical parameter, especially when the selectivity is low.(2,4)

It has been shown, that the region where the bands interfere is strongly controlled by the column efficiency. With increasing the column efficiency, the bands in the interfering region become narrower and narrower, thus providing less overlap.(5) When recovery yield is constrained, the higher column efficiency is required as well. Therefore, in order to produce better separations between epirubicin and doxorubicin, the two parameters influencing the column efficiency, i.e., the column temperature and the flow-rate of mobile phase should be considered and optimized, consecutively, for a given column.

The data obtained during the optimization of these parameters are summarized in Table 1. At room temperature (18°C) and a flow-rate of 0.8 mL/min, the retention time and the theoretical plate of epirubicin were 27.5 min and 24500, respectively. With increasing the temperature to 40°C and holding all other parameters constant, the column efficiency was increased to 37060 and retention time was decreased from 27.5 min to 16.2 min. Although the maximum operating temperature of the column permitted is 60°C according to the instruction of manufacturer, all the preparative operations were run at 40°C in our study, to reserve stability of both the sample and the column under the overloading conditions.

With reducing the flow-rate to 0.4 mL/min, the column efficiency was further increased and resolution reached 2.9. A flow-rate of 0.25 mL/min resulted in even higher resolution (3.0), but the lower flow-rate led to longer separation time and the decrease of production rate. Therefore, 0.4 mL/min was used in this work.

The condition, which resulted in higher resolution and column efficiency finally, is methanol-water (50:50, v/v, pH=2.4, adjusting pH with formic acid) at a flow-rate of 0.4 mL/min and 40°C. This is a trade-off between column efficiency and production rate.

Preparative Separation

A traditional way to optimize the load in preparative chromatography is called touching-band optimization, in which the injected quantity of the sample to be purified is usually increased until the peaks of the compound of interest and some other compounds touch.(6) This optimization approach has the advantage of being quite simple, 100% purity, and higher recovery, but the maximum

injected quantity would be much smaller, therefore, the production rate is low. Especially, it prevents taking advantage of specific effects that happen at larger loads. These effects concern the displacement of one product by another one. Under the condition of sample overloading, the components in the sample interfere and the elution peaks are modified due to the effect of competitive adsorption behavior on the surface of the stationary phase. The higher retained component pushes the lower ahead of it and the separation is actually much better than could be expected.

An overloading study was performed to determine the optimal injection volume of the sample solution. 10, 20, 40, and 80 μL, the solution of raw product without dilution, were injected, respectively. With increasing sample size, the retention time of the epirubicin peak became shorter and shorter; the peak shapes of epirubicin were gradually changed from symmetric to triangular, and the rear boundaries of the peaks corresponding to different loading overlap together at the same time (Fig. 4). These facts have indicated that the adsorption isotherm of

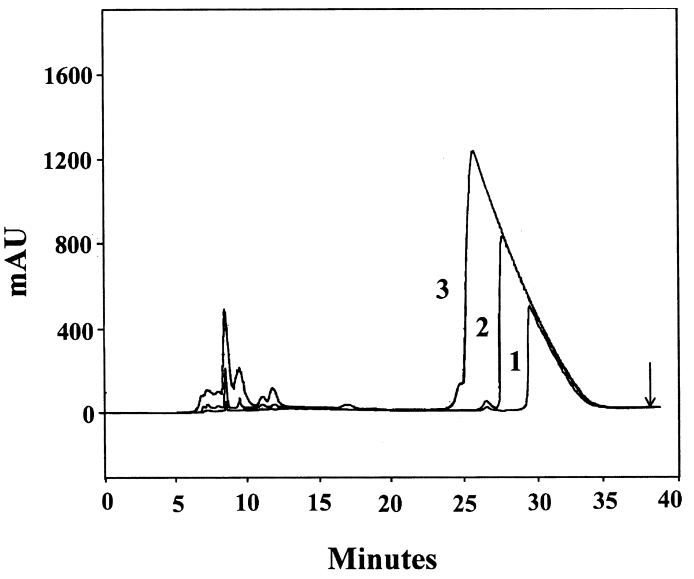

Figure 4. The changes of epirubicin peak shape with increasing the sample load of (1) 20 μL, (2) 40 μL, and (3) 80 μL raw product without dilution. Mobile phase: methanol-water (50:50, v/v, pH=2.4). Flow-rate: 0.4 mL/min; Column temperature: 40°C. Wavelength: 280 nm. The arrow indicates the point where the column is washed with methanol.

epirubicin in the separation conditions is Langmuirian. Doxorubicin was always eluted before epirubicin under the overloading condition with different amounts of sample injected, due to the displacement effect caused by Langmuir adsorption behavior of the sample. So the overlap band strategy was used in our study. This strategy has a special advantage for improving the production rate of the interested compound eluted after the major impurity, because displacement effects are predominant and the production rate can be raised three times in a large sample load.(7-9) In this study, 80 μL of the raw product solution was injected per run. In order to prevent detector saturation, the wavelength at 280 nm, instead of the maximum adsorption wavelength, was selected for monitoring.

The fraction collection can be exerted according to the retention time or the signal level of the detector. In our study, the collection cut points of the fractions were made on the basis of the detector response rather than retention time. Therefore, even if a little change of the separation or retention time occurred, the purity of the fractions could be maintained.

Fig. 5 illustrates collection of fractions by the optimal cut points. The horizontal dashed line indicates the starting points A, B, and C, corresponding to the responses of 0, 400, and 800 mAU, respectively. The vertical dashed line indicates the end point D at 100 mAU. Fraction I (from A to B) and II (from B to D), are shown in Fig. 5a, and fraction III (from C to D) in Fig. 5b. They were all collected for further purity analysis. Fraction I was a mixture of epirubicin and doxorubicin, which showed that doxorubicin was not removed at all from the raw product of epirubicin. The purities of epirubicin in fraction II and fraction III were found to be 99.5% and 100%, respectively. The corresponding recovery yields were 90% and 60%, respectively.

These results indicate that the recovery yield was decreased dramatically, while the purity of epirubicin was increased. Theoretically, the production rate of the more retained compound increased rapidly while decreasing the required purity, when the compound is rich in the feed.(7,8) Therefore, a compromise between the purity and the yield of epirubicin has to be made. The eluted epirubicin was collected from 400 mAU (B) to 100 mAU (D). After the collection was completed, the column was washed with methanol for 5 min at 1 mL/min to elute other impurities strongly retained on the column, and then re-equilibrated with the initial mobile phase for 10 min.

Analytical chromatograms of the collected fractions are shown in Fig. 6. Figure 6a is the chromatogram of a mobile phase blank. Compared with the raw product, in which 85% of epirubicin was contained, the purity of the final product was rapidly increased to greater than 99% (Fig. 6b). Good reproducibilities were observed by continuously repetitive injections.

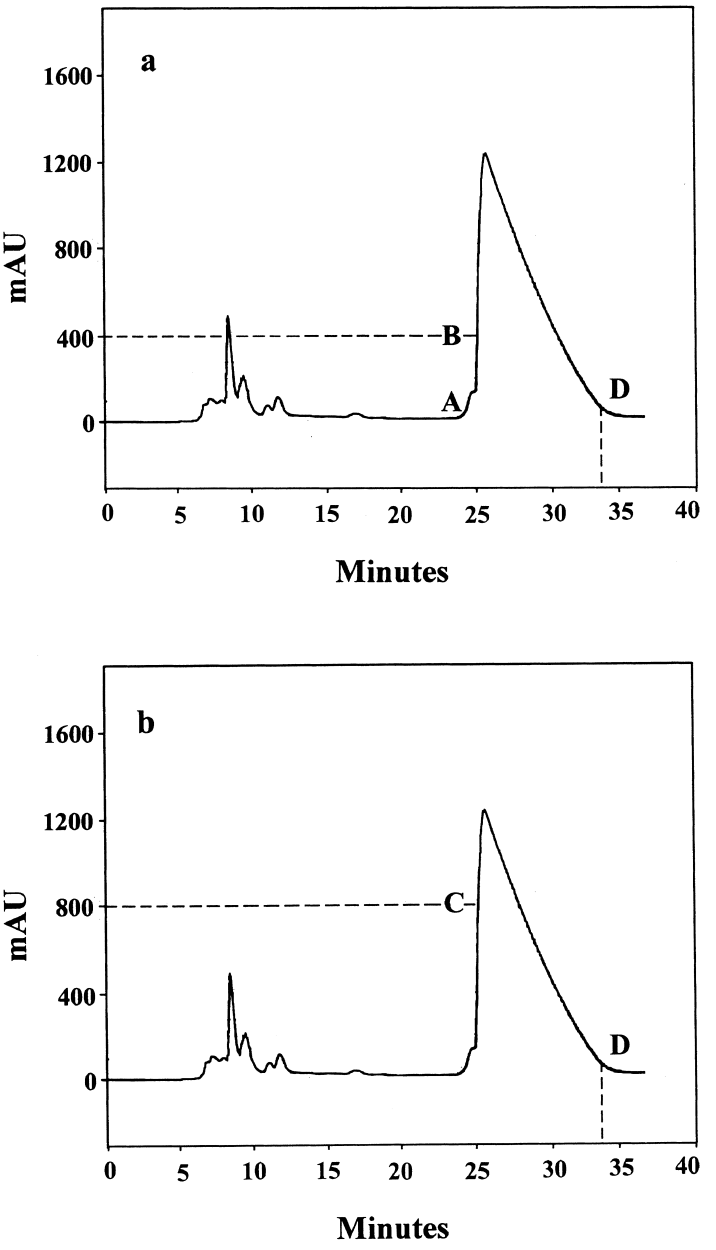

Figure 5. Preparative chromatogram with the selection of cut points for the collection of epirubicin. The horizontal dashed line indicates the starting cut points A, B, and C, corresponding to the response at 0, 400, and 800 mAU, respectively. The vertical dashed line indicates the end point D corresponding to the response at 100mAU. (a). Collections of Fraction I and II; (b). Collection of Fraction III. Other conditions as given in Experimental section.

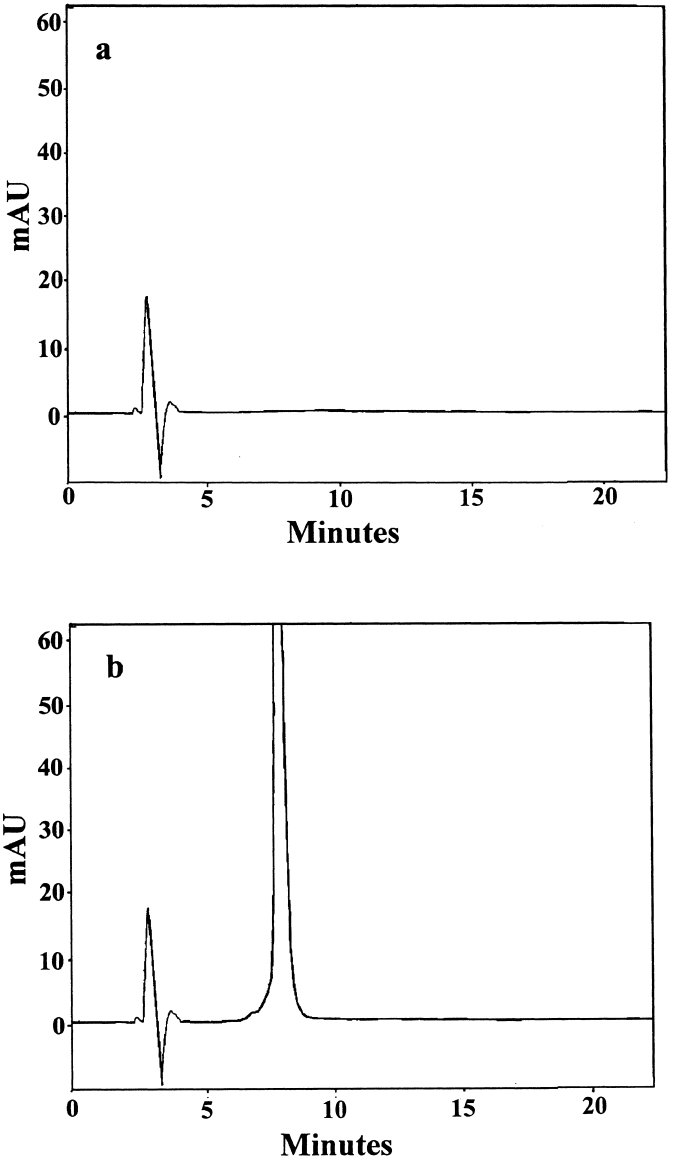

Figure 6. Test for the purity of the collected epirubicin. (a). Mobile phase blank; (b). Fraction of purified epirubicin. Other conditions as given in Experimental section.

CONCLUSION

We have developed a rapid isocratic preparative HPLC to purify epirubicin from a raw product, to which little attention has been paid in literature. Overlap band strategy is a good choice for epirubicin, having Langmuir adsorption behavior, under experimental conditions. A satisfactory result, with the purity of greater than 99% and the recovery of 90% for epirubicin, was achieved by using methanol-water (50:50, v/v, adjusting pH to 2.4 with formic acid) as a mobile phase on a reversed phase C_{18} column at 0.4 mL/min and 40°C. The method is reproducible and reliable, and could be used as a guide to scale up the industrially preparative separation of epirubicin.

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