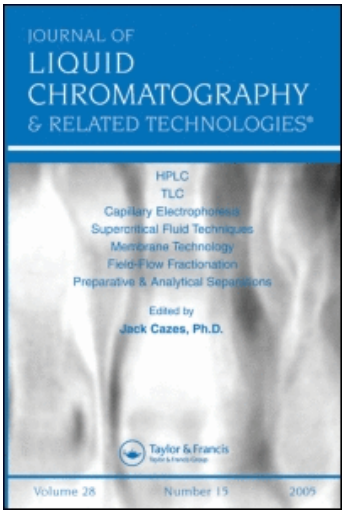


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# Isolation and Purification of Epirubicin from Raw Product by Preparative Chromatography on a Silica Column with Aqueous-Rich Mobile Phase

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# Isolation and Purification of Epirubicin from Raw Product by Preparative Chromatography on a Silica Column with Aqueous-Rich Mobile Phase

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**Abstract:** A novel chromatographic method has been developed for the preparative separation of epirubicin from raw product solution in overloaded elution mode using an analytical silica column with aqueous-rich mobile phase. The retention behavior of sample solutes was investigated in order to optimize the resolution by changing the mobile phase composition, flow-rate, and column temperature. It has been found that the high-purity silica exhibits typical reversed-phase behavior in a highly aqueous environment with little or organic modifier, and the nature of the organic modifier has little effect on its hydrophobic property. More significantly, the overloaded peak profiles of the solutes under such conditions were right-angled triangle with the rear of the peaks in coincidence, a typical overloading peak profile belonging to competitive Langmuir isotherm. The preparative separation was optimized by adjusting the sample size and detecting the sample at its low UV absorbance, and the positions of the cut points were determined by the detector response values. Even with an $\alpha$ value as small as 1.04, satisfactory preparative separations were observed by effectively utilized displacement effects and touching-band optimization. The recovery of epirubicin was 90% and the purity was more than 99% as measured by RPLC on a $C_{18}$ column. The added stepwise elution was a crucial

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procedure since it not only promoted the separation time but also regenerated the silica column to prolong its lifetime.

**Keywords:** Preparative chromatography, Mobile phase composition, Optimization, Epirubicin, Antibiotics



## INTRODUCTION

Today, reversed phase liquid chromatography (RPLC) has become the most useful liquid chromatography technique. This is due to the fact that RPLC has a broad range of applications. However, new separation challenges have arisen from the constantly developing practical applications to the analysis of basic solutes. For such highly polar compounds, very weak mobile phases must be used to promote retention and separation. These high or 100% aqueous mobile phases for desired separation can cause the collapse of high density $C_8$ or $C_{18}$ chains on the surface of silica as well as poor spraying condition and sensitivity for MS.[1,2] Also, severe peak tailing is often observed during the development of basic drugs.[3,4] This is why drug metabolism, drug discovery, and combinatorial chemistry scientists have turned to using silica column and aqueous-organic mobile phase for solving these challenging separation problems.[1,2]

The chromatography of bases on unmodified silica with reversed-phase conditions was published as early as the 1970's.[5] Since then, this chromatographic system has received much attention and has been used extensively for various types of compounds.[1–3,6–12] Many polar basic compounds have been separated with good peak symmetry and high efficiency on silica, either with organic-rich mobile phases (i.e., the so-called hydrophilic interaction chromatography, HILIC)[1–3,9,11] or with aqueous-rich eluents (i.e., the so-called psedo-reversed-phase chromatography).[6,7] Recent years have seen a great amount of reports about the former in bioanalytical LC-MS applications,[2,13–15] but few studies have been devoted to the applications of the latter. However, the benefits of the latter, such as the use of mostly aqueous mobile phases and a comparatively inexpensive and stable column, make the application of this separation mode quite attractive. For instance, it has the great potential to be used as a low-cost purification process option for basic compounds, but there is not enough information available in the literature about this system, its operation, and its commercial feasibility, etc.

Epirubicin (4′-epidoxorubicin) is an anthracycline antibiotic and antitumour derivative of doxorubicin, with potent activity against a wide range of tumors. It differs from doxorubicin in the inversion of stereochemistry at C-4′ (daunosamine ring). Doxorubicin, together with daunorubicin and epidaunorubicin, are the major impurities of epirubicin. In our previous works, RPLC has been applied to isolate and purify epirubicin from its impurities in the overloaded and displacement modes, respectively.[16,17] Recently, we

have investigated the chromatographic behavior of these four analogues on high-purity silica in HILIC mode.[18] This paper illustrates a practical example of the application of the silica column with aqueous-rich eluents for the isolation of basic compounds. Its purpose is not to undermine the dominant position of reversed-phase separations but to provide an alternative for highly polar basic compounds.

## EXPERIMENTAL

### Apparatus and Reagents

The TSP chromatograph (Thermo Separation Products, San Jose, CA, USA) consisted of a SpectraSYSTEM P4000 pump, a SpectraSYSTEM AS3000 autosampler with a fixed-loop injection valve, 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). The development of both the analytical method and preparative separations were performed on a Kromasil KR100-5SIL column, 250 mm × 4.6 mm I.D. (Eka Chemicals AB, Bohus, Sweden). A Kromasil 100-10C18 column (250 mm × 4.6 mm I.D., 10 μm) also from Eka Chemicals was employed for the purity analysis of the fractions. All solvents and reagents used for method development and the purity analysis were of HPLC grade. Water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Epirubicin raw product solution, epirubicin, doxorubicin, daunorubicin, and epidaunorubicin standards were obtained as a gift from Hisun Pharmaceutical Inc. (Zhejiang, China). The standards were used to characterize the target compound and impurities in the method development and the fraction analysis.



### Experimental Procedure

Preparative separations were performed on a Kromasil KR100-5SIL column (250 mm × 4.6 mm I.D.) using acetonitrile-ammonium formate buffer (pH 2.9, 20 mM) (5:95, v/v) as mobile phase at ambient temperature. The flow-rate was 0.65 mL/min. Epirubicin (40 μL) raw product solution without dilution was manually injected into the column with a syringe. The eluted epirubicin from the column was monitored at 320 nm and collected according to the response level of the detector. After the collection was completed, the ratio of acetonitrile in mobile phase up to 40% (v/v) was increased, and the column washed for 10 min at 1.0 mL/min to remove all impurities strongly retained on the column. Then, the column was re-equilibrated with five column volumes of the initial mobile phase before the next separation.

Each fraction was analyzed for purity on a Kromasil 100-10C18 column at room temperature. The middle fraction containing a high concentration of epirubicin was diluted 10 fold with the mobile phase and 20 μL was injected. Of the earlier and later collected fractions, 20 μL was analyzed without dilution. The mobile phase was methanol-water (60:40, v/v, pH adjusted to 2.4 with formic acid). The flow-rate was 1.0 mL/min and the detection was at UV 254 nm, the maximum absorbance wavelength of epirubicin.

## RESULTS AND DISCUSSION

### Overview of the Retention Behavior of the Solutes

In order to examine the retention behavior of epirubicin and its impurities, the chromatography was carried out on the Kromasil SIL and $C_{18}$ columns under various reversed phase conditions. Table 1 summarizes the obtained data. It has been found that the retention increases with the raising hydrophobic character of the solutes, and the elution order of the four compounds on the silica column is consistent with that obtained from RPLC on $C_{18}$ column,[16] but opposite to that obtained with aqueous-rich eluents with same silica column.[18] This result reveals that the unbonded high-purity silica exhibits typical reversed phase chromatographic behavior in a highly aqueous environment, with none, or a small amount of organic modifier present. However, silica acts as a weak reversed-phase packing, showing relatively lower separation selectivity compared to $C_{18}$. This chromatographic



***Table 1.*** Comparison of retention behavior of epirubicin and its impurities on silica versus $C_{18}$ column

| | $C_{18}$ (10 μm)[a] | | Silica (5 μm)[b] | | Silica (5 μm)[c] | |
|---|---|---|---|---|---|---|
| Compound | k′ | Plates/m | k′ | Plates/m | k′ | Plates/m |
| Doxorubicin | 1.43 | 10255 | 2.95 | 2758 | 0.71 | 15743 |
| Epirubicin | 1.69 | 11735 | 3.04 | 2829 | 0.73 | 16416 |
| Daunorubicin | 3.51 | 15544 | 5.74 | 2948 | 1.04 | 17052 |
| Epidaunorubicin | 4.38 | 15569 | 5.84 | 3053 | 1.06 | 17271 |

The Kromasil columns with dimension of 250 × 4.6 mm I.D. were obtained from Eka Chemicals. The pore size of all packings is 100Å and particle sizes are indicated in the table. UV detection: 254 nm. Injection: 20 μL.

[a]Mobile phase: methanol-water (60:40, v/v, adjusting pH to 2.4 with formic acid), flow-rate: 1.0 mL/min. The solutes are injected in mixture.

[b]Mobile phase: sodium formate buffer (20 mM, pH 2.9), flow-rate: 1.0 mL/min. Solutes of 0.025 mg/mL were injected singly.

[c]Mobile phase: sodium formate buffer (20 mM, pH 2.9)-acetonitrile (95:5, v/v), flow-rate: 1.0 mL/min. Solutes of 0.025 mg/mL were injected singly.

behavior of high-purity silica is essentially in agreement with similar findings on a conventional, more acidic and less purified silica.[3]

Also, the dependence of retention on the sample size of epirubicin and its impurities with aqueous mobile phase has been examined. Reported in Figure 1 are the typical profiles of epirubicin. With pure aqueous buffer (pH 2.9) as mobile phase, peaks become increasingly right-triangular in shape, and retention times are decreased with the peak tails in coincidence when increasing sample size. It quite resembles the profiles obtained for compounds having a Langmuir isotherm.[19] Moreover, the impurities exhibit a close similar behavior to epirubicin. This phenomenon is also like that on $C_{18}$ in RPLC;[16] thus, there seems no fundamental difference to some extent in the properties between $C_{18}$ and unmodified silica in aqueous-rich mobile phases.



## Optimization of Operation Conditions

### Selection of Mobile Phase Composition

The mobile phase composition was optimized to provide a reasonable retention factor for epirubcin and the best selectivity for the separation of epirubicin from its impurities. The influence of the type and the concentration of the polar organic modifier in the eluent, as well as the pH and concentration of

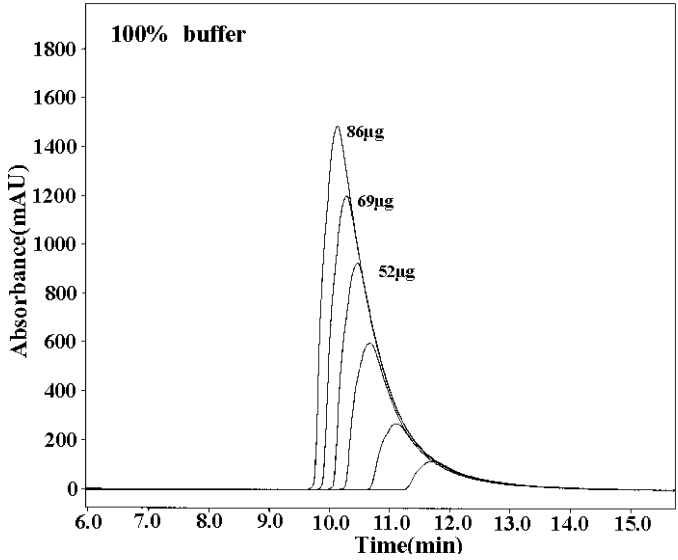


*Figure 1.* The changes of epirubicin peak shape with increasing the sample size. Mobile phase: sodium formate buffer (20 mM, pH 2.9), flow-rate: 1.0 mL/min. UV detection: 280 nm.

the buffer on the solute retention and selectivity could be expected on bare silica under aqueous-rich eluents, as in ordinary reversed phase chromatography. Therefore, the retention behavior of the solutes was investigated as a function of these parameters to find the optimum experimental conditions. The results have indicated:

Mobile phases containing the same concentration (5%, v/v) of methanol, acetonitrile, tetrahydrofuran (THF), or isopropanol could not completely separate epirubicin from doxorubicin, but doxorubicin was always eluted before epirubicin in all cases, and the retention order is consistent with that obtained from RPLC on a $C_{18}$ column.[16] Moreover, the selectivity does not change appreciably by changing the type of modifier (Table 2), indicating that similar retention can be achieved with any of these solvents when used at the appropriate concentration. It can be seen in Table 2 that the elution strengths of the various organic solvents are parallel to their usual reversed-phase behavior. Methanol was the strongest solvent of the four solvents tested, its strength for the solutes eluting is the weakest. This result also implies that the nature of the organic modifier has little effect on the hydrophobic property of silica under aqueous-rich conditions. Figure 2 shows the typical comparison of chromatograms obtained with acetonitrile and methanol as examples. From Table 2 and Figure 2, acetonitrile was selected as the organic modifier due to a compromise between retention time, resolution, and column efficiency.

Increasing the content of organic modifier decreases the retention times of the solutes as well as the resolutions. An increase of acetonitrile in 20 mM formate buffer at pH 2.9 from 20% to 50% (v/v) had pushed the solute peak into the solvent front, and the solutes were eluted without discrimination. With a mobile phase containing no organic modifier, the separation will be increased; however, the overall separation time can also be increased. Furthermore, the larger the retention time, the lower the concentration of the fractions collected in the elution mode because of axial dispersion. Thus, 5% (v/v) acetonitrile was chosen due to its short separation time and reasonable selectivity.

***Table 2.*** Effect of organic modifier on the chromatographic parameters of epirubicin. Mobile phase: sodium formate buffer (20 mM, pH 2.9) modified with various organic solvents (95:5, v/v). Flow-rate: 1.0 mL/min. UV detection at 254 nm

| Organic modifier | $t_R$ (min) | As | $\alpha^a$ | $Rs^a$ | Plates/meter |
|---|---|---|---|---|---|
| Methanol | 6.448 | 1.94 | 1.03 | 3.37 | 12586 |
| Acetonitrile | 5.16 | 1.55 | 1.03 | 2.77 | 16459 |
| THF | 4.558 | 1.69 | 1.03 | 1.78 | 12666 |
| Isopropanol | 4.952 | 1.69 | 1.02 | 1.93 | 12217 |

[a] $\alpha$ is separation factor between epirubicin and doxorubicin; Rs is resolution between epirubicin and daunorubicin.

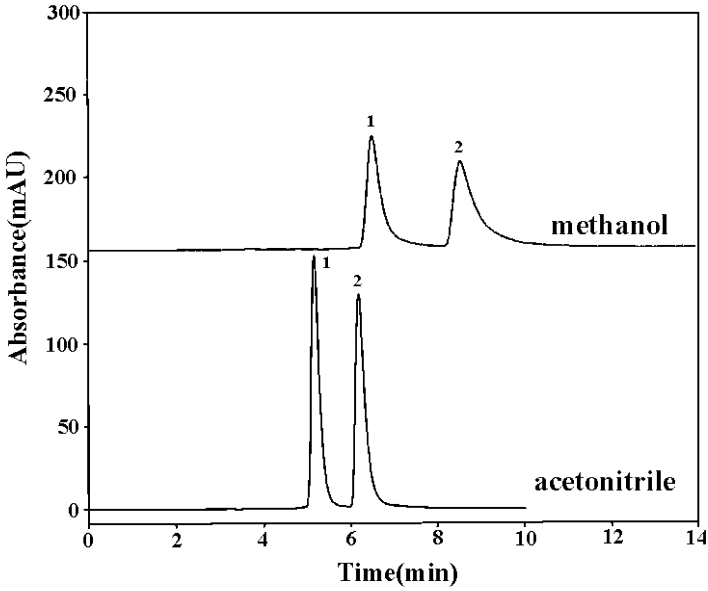


***Figure 2.*** Typical chromatograms of epirubicin and its impurities with various organic modifiers. Conditions: Kromasil 100-5SIL (5 μm); mobile phase: 5% (v/v) methanol or acetonitrile in sodium formate buffer (20 mM, pH 2.9); flow-rate: 1.0 mL/min; injections: 20 μL; UV detection: 254 nm. Peaks: (1) doxorubicin + epirubicin, (2) daunorubicin + epidaunorubicin.



In order to simplify the post-purification handling, ammonium formate was employed to provide both the eluent ionic strength and pH environment instead of sodium formate, though the latter produced higher column efficiency and resolution. As the buffer pH is increased, the peak symmetry is increasingly impaired, and accompanied by a reduction of column efficiency as well as resolution. Buffers at pH $>3.6$ have made solutes form severely broad peaks and spoiled the separation. So, the acidic buffer was selected to keep the solutes and the majority of silanol groups on silica surface in unionized forms and to make the separation mechanism dominantly hydrophobic. Here, pH 2.9 was utilized since it produced reasonable resolution and acceptable peak shape. The investigation of the effect of ionic strength at pH 2.9 on retention indicates that the retention of the solutes has been slightly decreased with an increase of the buffer concentration, but no salt in the mobile phase has led to poor reproducibility. As a result, 20 mM was found to have adequate buffering capability, and very high stability and reproducibility of retention of the solutes could be obtained. These observations have also indicated that the retention mechanism of the solutes is a combination of ion-exchange and hydrophobic retention.

Selection of the Flow Rate and the Column Temperature

In the elution chromatography, resolution suffers as the peak width and loading increase. In order to increase the production rate of each cycle, high column efficiency is the critical parameter, especially when the selectivity is low.[20,21] It has been shown that, when the column efficiency increases, the peak bands are narrower, providing greater a region to broaden the bands by increasing the sample load.[22] In order to increase column efficiency and, thus, the resolution, the flow-rate of mobile phase and the column temperature have been optimized consecutively.

Table 3 summarizes the chromatographic data obtained during the process of optimization. At room temperature (20°C), a decrease of flow-rate from 1.0 to 0.35 mL/min led to an increase of column efficiency and resolution between epirubicin and its latter eluted impurities, but had a slight improvement in the separation factor between epirubicin and doxorubicin. Though a flow-rate of 0.35 mL/min produced a higher resolution (2.72) between epirubicin and the late-eluting impurities, the lower flow-rate resulted in longer separation time and the decrease of production rate. In consideration of both runtime and resolution, 0.65 mL/min was used in this study. It can be seen from Table 3 that the column efficiency, solute retention, and the resolution are improved insignificantly with column temperature rising, so, all separations were carried out at room temperature for convenience.

The optimum condition for the separation is acetonitrile-ammonium formate buffer (20 mM, pH 2.9) (5:95, v/v) at a flow-rate of 0.65 mL/min and ambient temperature. This is a reasonable compromise between separation selectivity, separation time, and column efficiency.



## Preparative Separation

In the development of chromatographic systems for pharmaceutical or biomedical separations it is important not only to produce the most efficient purifi-

***Table 3.*** Summary of chromatographic data. Mobile phase: ammonium formate buffer (20 mM, pH 2.9)-acetonitrile (95:5, v/v). UV detection at 254 nm

| Fow-rate (mL/min) | $t_R$[a] (min) | Temperature (°C) | $\alpha$[a] | Rs[a] | Plates/meter |
|---|---|---|---|---|---|
| 1.0 | 6.473 | 20 | 1.03 | 2.28 | 10,234 |
| 0.65 | 10.230 | 20 | 1.04 | 2.53 | 10,796 |
| 0.35 | 19.217 | 20 | 1.04 | 2.72 | 12,522 |
| 0.65 | 10.164 | 30 | 1.04 | 2.53 | 10,935 |

[a] $t_R$ is retention time of epirubicin; $\alpha$ is separation factor between epirubicin and doxorubicin; Rs is resolution between epirubicin and daunorubicin.

cation system, but also to find the conditions under which the required purity of the target compound is achieved with minimum expenditures.[23] Although epirubicin still could not be completely resolved from doxorubicin on silica by any of the reversed-phase eluents that we tried, the above-selected experimental condition enabled baseline separation of epirubicin from most late-eluting impurities in the raw product. Moreover, the stable and comparatively inexpensive column packing, low-cost eluents, and relatively short separation time are the preferred conditions required in preparative chromatography. Thanks to the small amounts of doxorubicin in the raw product (Figure 3), it is possible, in practice, to optimize the isolation by effectively coupling the advantages of displacement effects and touching-band optimization, while the desired compound is achieved at the required purity.[22,24,25] We expected that the earlier eluting doxorubicin should be largely removed by the displacement effects, and the later eluting daunorubicin and epidaunorubicin, etc., should be removed by the touching-band optimization.

The injection volume of the sample solution is an essential parameter to be optimized in this case. The raw product solution (10, 20, 40, and 60 μL) without dilution were injected, respectively. Column effluent was monitored at 320 nm instead of 280 nm, at which 10 μL of sample solution saturated the detector. Effluent fractions of 0.2 mL were collected during the separation



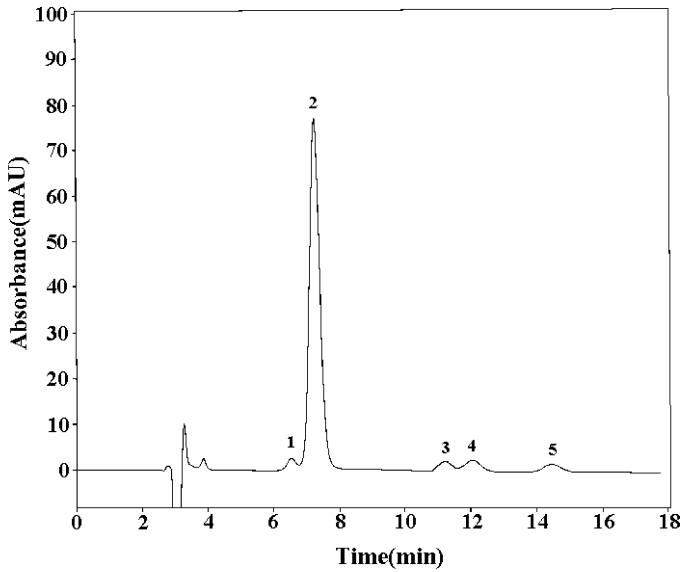


***Figure 3.*** Chromatogram of raw product (diluted 80 fold with mobile phase). Conditions: Kromasil 100-10C18 (10 μm); mobile phase: methanol-water (60:40, v/v, pH 2.4 adjusted with formic acid); flow-rate: 1.0 mL/min; injections: 20 μL; UV detection: 254 nm. Peaks: 1 = doxorubicin, 2 = epirubicin, 3 = unknown impurity, 4 = daunorubicin, 5 = epidaunorubicin.

and subsequently analyzed by RPLC on a $C_{18}$ column. As the sample load was increased, it gave rise to shorter retention time and a triangular peak shape of epirubicin similar to Figure 1. Fraction analysis demonstrated that doxorubicin was always eluted ahead of epirubicin with different quantities of sample injected, due to the displacement effects. The separation is actually much better than could be expected. Touching bands did not take place with 20 μL of sample solution injected. There is a little degree of overlap of the two bands with the injection volume of 40 μL. Detector saturation occurred with the injection volume up to 60 μL. More importantly, the impurities eluted after epirubicin was pulled into the major peak owing to tag-along effect, and the desired purity of epirubicin was obtained with a low recovery. Taking both of the resolution and recovery into consideration, a good compromise was reached at an injection volume of 40 μL.

The positions of fraction cut points were selected on the basis of the detector response values rather than retention time, to maintain the purity of the fraction.[16] Figure 4 shows the optimal cut points. The points A, B, C, and D correspond to the responses of 20, 400, 700, and 20 mAU. Chromatograms of the fractions I (from A to B), II (from B to C), and III (from C to D) are presented in Figure 5. Fraction I contained a small amount of doxorubicin (Figure 5b). The purity of epirubicin in Fraction II was 99.3%, and no detectable amounts of daunorubicin and epidaunorubicin were in this



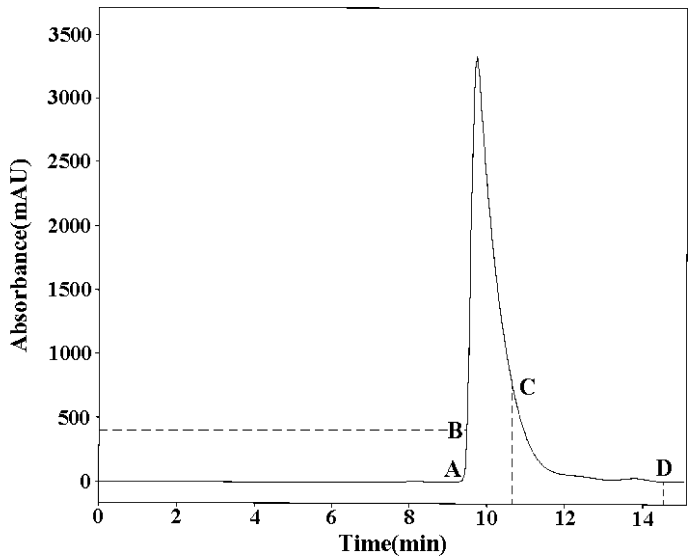


***Figure 4.*** Preparative chromatogram with cut points. The cut points A, B, C, and D are corresponding to the responses of 20, 400, 700, and 20 mAU. Fractions from A to B, B to C, and C to D were collected for further purity analysis. Other conditions as given in Experimental section.

fraction (Figure 5c). Fraction III was seen to contain a considerable amount of the late-eluting impurities (Figure 5d). The overall recovery of purified epirubicin per cycle was 90%. These results demonstrate that preparative separation of epirubicin is feasible when the silica column is operated under aqueous-rich mobile phase according to the principles of non-linear chromatography.

In order to reduce band broadening and elute the strongly retained substances completely, stepwise elution was employed, in which the changes in the organic concentration of the eluent (from 5% to 40%, v/v) and the flow-rate (from 0.65 to 1.0 mL/min) were introduced into the column simultaneously. Stepwise elution was started at the point C in Figure 4 where the collection of the target compound was completed. Figure 6 illustrates the starting and ending points of stepwise elution. For comparison, the chromatogram as obtained under conditions without stepwise elution is also included. Apparently, the band became narrower compared to the band without stepwise elution, and Fraction III was concomitantly concentrated. The strongly retained compounds can be readily removed from the



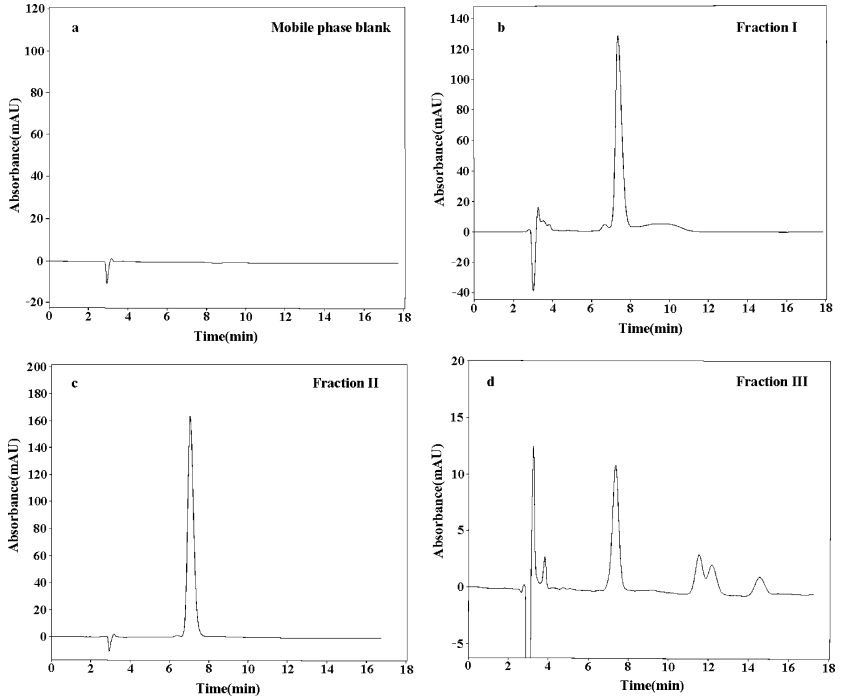


***Figure 5.*** Test for the purity of the collected fractions. (a) Mobile phase blank; (b) 20 μL of Fraction I; (c) 20 μL of Fraction II diluted 10 fold with mobile phase; (d) 20 μL of Fraction III. Other conditions as given in Experimental section.

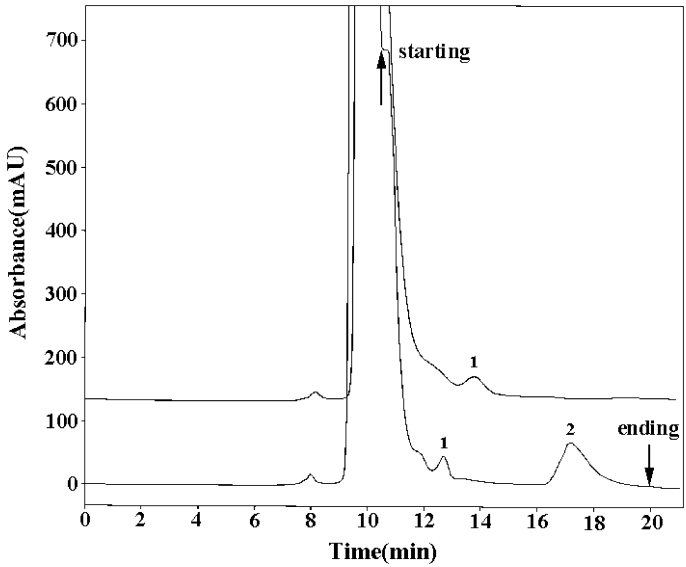


***Figure 6.*** Preparative chromatograms with (the lower one) and without (the upper one) stepwise elution. The ↑ indicates the starting point of stepwise elution, where the column is washed with ammonium formate buffer (20 mM, pH 2.9)-acetonitrile (60 : 40, v/v) at a flow-rate of 1.0 mL/min. The ↓ indicates the ending point of stepwise elution, where the column is re-equilibrated with initial mobile phase. 1 = unknown impurity; 2 = the strongly retained compounds.



column. When the undesired analogs and strongly retained impurities were washed out (peak 2 in Figure 6), the column was re-equilibrated with 5 column volumes of the initial mobile phase prior to the beginning of the next separation.

Generally, silica is more reactive compared to reversed-phase stationary phases. Irreversible adsorption of sample components is subject to occur, which can lead to column deterioration. In fact, the stepwise elution is of practical importance in this case, since it is an efficient way to wash the strongly retained impurities without loss of resolution. The silica column has been used for at least six months without significant loss of its separation efficiency and capacity following stepwise elution. The time for washing is short and small amounts of solvent are required during re-equilibration. Accordingly, the decrease of the production rate remained modest. Here, it is advisable to use stepwise elution as an effective regeneration method to prolong the column lifetime, rather than a fast purification and concentration method for epirubicin. In addition, no evidence indicated the dissolution of the packing over 30,000 column volumes of mobile phase passing through the column.

## Comparison with a Conventional RPLC Method

A comparative evaluation of the performance of the method presented in this study was made with the previously reported conventional RPLC method on a $C_{18}$ column,[16] which was shown in Table 4. In the RPLC method, the sample loading was comparatively large due to the high separation factors between the solutes; thus, obtaining high throughput and yield. This is a distinct advantage of the conventional RPLC method over the method in this work. The low separation power may be an intrinsic drawback of silica under aqueous-rich eluent and largely limit its application in practice. But in the present study, a reasonable amount of epirubicin was achieved at high purity and recovery. In spite of the low sample loading, the overall cycle of this technique, including separation and washing procedure was completed in about 20 min, less than half the run time of the RPLC method, which can compensate the low production rate and yield to some extent. Furthermore, short separation time ensures higher product concentration in small fraction volumes, resulting in considerable savings of time in the subsequent treatment, e.g., for solvent removal. Also, the developed approach exhibited an obviously convenient feature, i.e., the use of room temperature and low



***Table 4.*** Comparison of this method with conventional RPLC method for the purification of epirubicin from raw product solution

| | This method | RPLC method |
|---|---|---|
| Column | Silica (250 mm × 4.6 mm, I.D.), 5 μm (Eka Chemicals) | $C_{18}$ (250 mm × 4.6 mm, I.D.), 10 μm (Eka Chemicals) |
| Mobile phase | Acetonitrile-ammonium formate bufffer (20 mM, pH 2.9) (5:95, v/v) | Methanol-water (50:50, v/v), pH 2.4 adjusted with formic acid |
| Flow-rate (mL/min) | 0.65 | 0.4 |
| Temperature (°C) | room temperature | 40 |
| Separation time | 20 min (including column regeneration) | >40 min (column regeneration not included) |
| Separation factor between epirubicin and doxorubicin | 1.04 | 1.3 |
| Injection volume of the raw product solution | 40 μL | 80 μL |
| Yield per run | 0.4 mg | 0.8 mg |
| Purity | >99% | >99% |
| Recovery | 90% | 90% |

viscosity of the eluent compared to the RPLC method. In addition, the relatively low cost of the column packing and eluent can be expected to significantly improve the economics of the purification process.

## CONCLUSION

This is the first report in the literature in which a bare silica column operated in the aqueous-rich mobile phase has been used for the isolation and purification of a basic drug from its raw product. Under the experimental conditions, the high-purity silica displayed extremely similar hydrophobic characteristics as conventional $C_{18}$ column, which were proved by the retention and overloading behavior of the solutes. The optimum condition for the separation of epirubicin was acetonitrile-ammonium formate buffer (20 mM, pH 2.9) (5:95, v/v) at a flow-rate of 0.65 mL/min and ambient temperature. The purity of epirubicin was >99% with the recovery of 90%. The developed method is a fairly economical process since it utilizes simple and inexpensive column packing and eluents. The availability of purification strategy should help industrial separation of epirubicin more efficiently and economically.



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