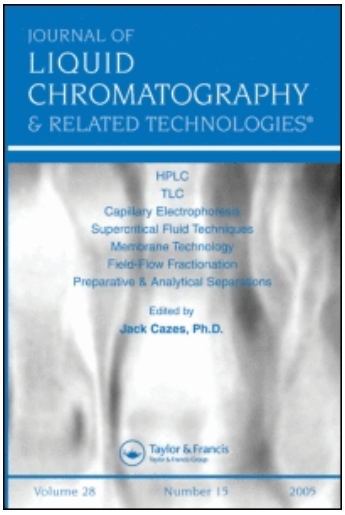

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Optimization Strategy for Purification of a Peptide from Complex Mixtures by Reversed-Phase Liquid Chromatography with a Back-Flushing Technique

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Abstract: An optimization strategy for the separation and purification of a peptide from crude material by reversed-phase liquid chromatography was developed. First, in the separation step, the tetrahydrofuran, instead of normal organic modifiers in the mobile phase, could improve the separation of the target peptide from its impurities; hence, the mass loading could be increased in each run. Furthermore, a back-flushing technique was introduced to elute the strongly retained impurities rapidly and economically. A raw mixture of 10 mg was purified on an analytical column by an overloading operation in one run, using a mobile phase consisting of tetrahydrofuran-water (30:70, v/v) + 0.1% TFA at a flow-rate of 1.0 mL/min and room temperature. The purity of the product was increased from 50% to 98% with recovery over 80%. The back-flushing step was very crucial to regenerate the system for the next run in the sense of both cost and time.

Keywords: Peptide, Separation, Tetrahydrofuran, Preparative chromatography, Displacement effect, Back-flushintg

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INTRODUCTION

With the rapid advances of biotechniques, more and more new biologically active peptides have been discovered and synthesized over the recent years. Since most of them contain closely related impurities, purification must be accomplished before the peptide product can be available commercially. At present, reversed phase liquid chromatography (RPLC) is generally accepted as a first choice of the methods for separation and purification of a peptide mixture due to its very high selectivity and efficiency.[1] As a rule of thumb, the mobile phase composition for RPLC separation of a peptide usually consists of organic solvents as modifier and organic acid additives as ion pairing agents. Normally, acetonitrile (ACN) is the favored organic solvent for most purposes. A more polar solvent (e.g., methanol, MeOH)[2] or a more nonpolar one (e.g., isopropanol, iPrOH)[3] may be required for separation of very hydrophilic or very hydrophobic peptides, respectively. Although, these solvent systems are efficient for most of peptide separations, they are not satisfactory enough to cope with more and more peptides with various structures and properties. Each case is specific and needs unique selectivity.

The special advantage of tetrahydrofuran (THF) for a change in selectivity was noted as early as 1978.[4] However, systematic examination of various experimental parameters and practical application examples for preparative purposes have rarely been reported.[5,6] The highlight of this work, is that the separation of the target peptide in THF contained mobile phase was dramatically improved in comparison with other organic modifiers, which was greatly beneficially to preparative work. As a result, we investigated the chromatographic behaviors in detail and optimized critical operating parameters, so that we can get a better understanding of the chromatographic behavior of the peptide in THF solvent system and an optimal separation condition.

EXPERIMENTAL

Apparatus and Reagents

The chromatographic system consisted of a SpectraSYSTEM P4000 pump, a SpectraSYSTEM AS3000 autosampler with a fixed loop injection valve, and a Spectra FOCUS diode array detector. TSP PC1000 Chromatography Manager software (3.0 version) was used for chromatographic system control, data acquisition, and chromatographic analysis. The column used for purification of the target compound was Kromasil C18 (10 μm, 100Å) with dimensions of 250 × 4.6 mm I.D. from Eka Chemicals AB, Bohus, Sweden. A six-port valve was mounted between injector and column for changing the direction of flow with the back-flushing technique, as shown in Figure 1. (In fact,

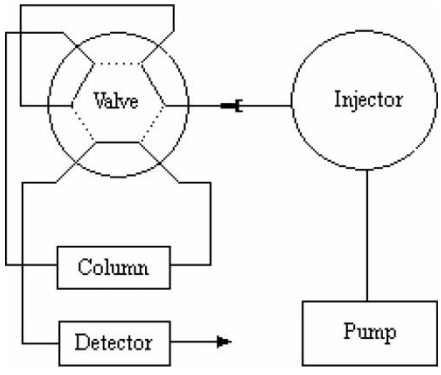

Figure 1. Schematic diagram of chromatographic purification process with back-flushing technique.

only a four-port valve was required, but because we couldn't find a suitable one, a six-port valve was used). The solid line in the valve indicates the flowing direction of mobile phase for separation of the target compound and the dashed line shows the flow of the back-flushing process for removing strongly retained impurities.

Acetonitrile, methanol, tetrahydrofuran, and trifluoroacetic acid (TFA) were HPLC grade purchased from Merck (Darmstadt, Germany). All other solvents and reagents were of analytical reagent grade. The water was prepared by the Milli-Q system (Millipore, Bedford, MA, USA.). The crude peptide sample produced by solid-phase synthesis was provided as a kind gift from Debai Pharmaceutical Inc. (Sichuan, China).

Experimental Procedure

Preparative separations were performed using THF-water (30:70, v/v) containing 0.1% TFA as mobile phase at ambient temperature. The flow rate was 1.0 mL/min. The fraction compounds eluted from the column were monitored at U.V. 280 nm. The crude sample was injected at the inlet of the column, at the beginning of the isolation process. Once the collection of the target compound was finished, the back flushing procedure was started immediately by turning the six-port valve. This operation allowed all impurities to be removed out of the column rapidly. After almost an equivalent time of the eluted target compound, the valve was turned back again for the next run.

For purity analysis, the mobile phase consisted of ACN-water (31:69, v/v) and 0.1% TFA. The detection of the peak was at U.V. 220 nm.

RESULTS AND DISCUSSION

Selection and Optimization of Organic Modifier

ACN, MeOH, and iPrOH are three organic solvents frequently applied in the separation and purification of peptides. In this work, THF was also tested as a modifier. The results were shown in Figure 2 (the concentrations of organic modifiers in the mobile phase were adjusted in order to give rational retention time, as well as adequate separation). Within the experimental time and sample size, the crude mixture contained a major peak (the percent of the major component was only 50%) and several impurities peaks produced during the synthesis. In order to purify the sample, the first step is to maximize separation factor (α) between the target component and any leading edge contaminants. In Figure 2, separation factors between target peak and the impurity before it were 0.87, 0.85, 0.88, and 0.69 in iPrOH, MeOH, CAN, and THF mobile phase, respectively. It's obvious that

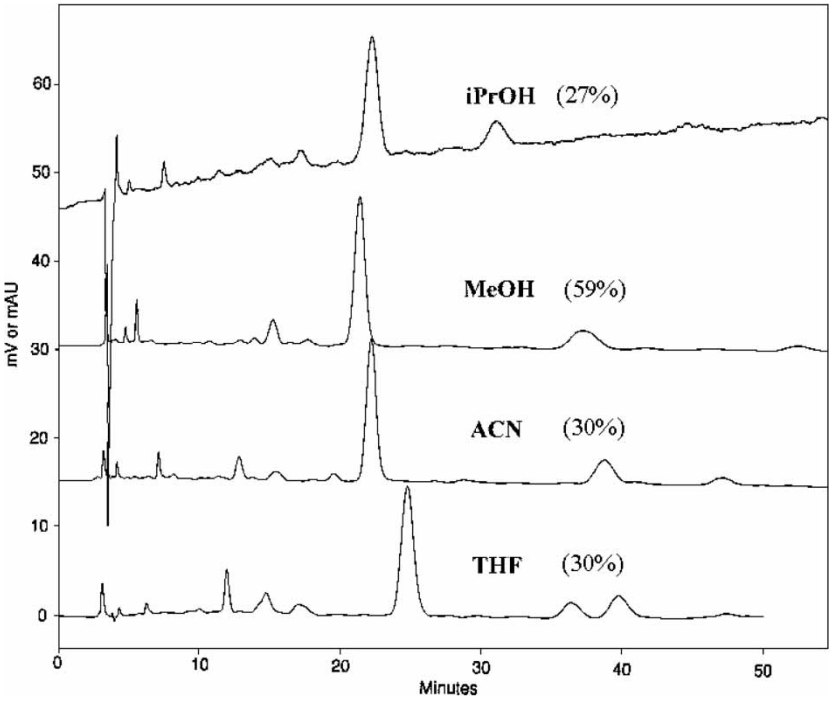

Figure 2. Chromatograms of test mixture of peptide obtained with different organic modifiers. (the contents of organic solvents in mobile phase shown in chromatograms). Conditions: Kromasil 100-10C18 (250 mm × 4.6 mm I.D.); mobile phase, water-organic solvents (containing 0.1% TFA); flow-rate, 1.0 mL/min; column temperature, 22°C; detection, UV at 280 nm; sample loaded, 5 μg.

an improved selectivity was found in the THF based mobile phase. It's recognized that a linear increase in production rate and a lograrithmic reduction in total cost can be obtained with increasing selectivity.[7] Thus, seen from the resolution between the main peak and the impurity before it, the separation in the THF system was more favorable. On the other hand, the latter eluted impurities became a little nearer to the main peak relative to MeOH and ACN solvent systems, but the band spacing (α value was 1.54) between them was wide enough for the desired isolation. As a result, the main peak resolved well with either of the neighbor interferences. Moreover, the peak shape was excellent, and the column efficiency was comparable with three other chromatographic conditions. In the present case, the THF system offers an attractive advantage and large scale preparative potential because of the higher selectivity, which also allows each individual sample to have its own personality; one separation system does not work well for all samples. Selectivity changes can be achieved by taking advantage of the hydrophobic diversity of peptides in different organic modifiers.

Further on, the influences of organic modifiers contents in mobile phase on solute retention were investigated. When different organic solvents were employed, the plots of logarithm retention factor (log k′) against the volume fraction of organic component (ψ) took on different trends, as shown in Figure 3. (Because iPrOH was analytical grade, the high volume in the mobile phase caused a poor baseline and failed determination. There was no data about it). The logarithm of the retention factor was a linear drop, with the increase of the MeOH volume fraction over the range investigated with correlation coefficients 0.9996. In contrast, marked deviation from linearity was found in either THF or ACN contained mobile phases. These differences attributed to the polarity diversities of different organic modifiers and their interactions with the stationary phase. Due to higher polarity of MeOH in comparison with ACN and THF, and lower affinity to the stationary phase, the probability of solvate shell formation on the stationary phase was lower.[8] Consequently, with a ψ increase, k′ reduced dramatically.

Within the interesting range of k′ in chromatographic optimization, e.g., $1 < k' < 20$,[9] as shown between two dashed line in Figure 3, a linear decrease in log k′ was obtained in ACN. On contrast, k′ decreased so slowly in THF that even when ψ reached 0.8, log k′ was still located in the favorable range. The flatter curve in THF means that the optimization of operating condition is easier in THF mobile phase. In the case where the slope of curve is steeper, the adjustment of mobile phase composition is more difficult to get an appropriate result. The results illustrated in Figure 3, together with those of the above discussion about the selectivity in different organic solvents, suggested that THF not only can afford improved selectivity for the peptide sample, but also its characteristic was favorable to the optimization of chromatographic separation. Through the study about the effect of different THF contents in mobile phase on the

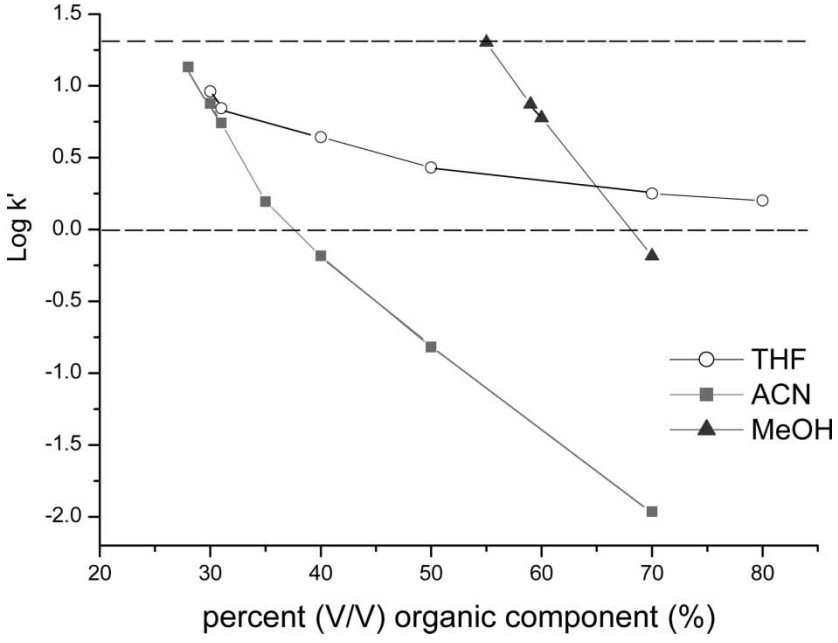

Figure 3. Logarithm of retention factors at different mobile phase compositions. The dash line indicated $k' = 20$ (top) and $k' = 1$ (bottom), respectively.

retention of solute, a level of 30% THF afforded an adequate baseline separation in a rational time.

Selection and Optimization of Organic Acid Additives

In RPLC, ion exchange (IEX) interactions between peptide and residual silanol groups on the surface of silica gel matrix cause band tailing.[10] Organic acids added to the mobile phase can prevent such interactions, and at the same time, increase the retention of the peptide through the forming ion pair.[11] In order to simplify the after treatment process of the collected fractions, three volatile organic acids including formic acid (FA), acetic acid (AA), and TFA were examined. Due to the weak ion pair capability of formic acid and acetic acid with charged peptides, the peptide was eluted close to the void volume. For the purpose of sufficient retention and baseline resolution, the concentration of THF in mobile phase was decreased to 12.5% and 20.5% with acetic acid and formic acid as additives, respectively, but the peptide was still eluted with tailing and the column efficiencies were poor (Table 1), whereas, TFA offered sufficient retain for the separation process. The reason is that TFA not only interacts

Table 1. Effect of organic acid addictive on the retention of peptide

Organic acids	t_R (min)	α	As	Plates/m
FA	19.8	0.80	1.32	10032
AA	20.3	0.85	1.30	10051
TFA	19.98	0.72	1.15	12954

At the sample pH (pH = 2), the concentration of FA, AA and TFA were 2.45%, 19%, 0.1%, in order to get comparable retention, the concentration of THF in mobile phase were adjusted to 20.5%, 12.5% and 31%, respectively. Other conditions are the same as given in EXPERIMENTAL section.

with the amino functions of the peptides via carbonyl group, but also acts as a proton acceptor via its fluoro atoms. Hydrogen bonding interaction existing between peptides and TFA is expected to reduce the virtual polarity of the peptides in acidic media.[12] Consequently, the retention of peptides was stronger with the addition of TFA, and no tailing was observed because of effective preventing IEX interaction.

Furthermore, we tested the effects of TFA concentration on chromatographic separation. Although, recently, some authors believed higher concentration of TFA (0.2% $\sim$ 0.25%) benefited the separation of peptides,[13] considering the duration of the column (used pH $\geq$ 2), the concentration range of TFA tested still covered 0.05% $\sim$ 0.1% (pH 2.3 $\sim$ 2.0). In general, within the experimental range, a case of higher concentration of TFA showed longer retention and less tailing. The optimum separation of impurities from the major peak was achieved with 0.1% TFA.

Effect of Column Temperature

The effect of temperature on the retention of peptides was illustrated in Figure 4. There was an interesting phenomenon—at 22°C, peak 1 and peak 2 were well resolved. As temperature was raised gradually, two peaks became nearer and nearer, and coeluted to a single peak at 45°C. Then, the two peaks were partially resolved again at 55°C, albeit with a reversal of elution order compared to 22°C. In addition, peak 1 was found to be almost temperature independent, other peptides all exhibited the trend of reducing retention time with elevated temperature. The results of temperature effect on the retention behavior indicated that owning to the different extents of temperature effect for different components, peptide selectivity can be partially changed by temperature. As for the target peptide, peak 4, the run time was shortened at higher temperature, but the resolution became poor. Moreover, a runs at higher temperature need more operation costs, and the biological sample took the risk of loss of its activity, so all separations were carried out at room temperature for process economics and feasibility.

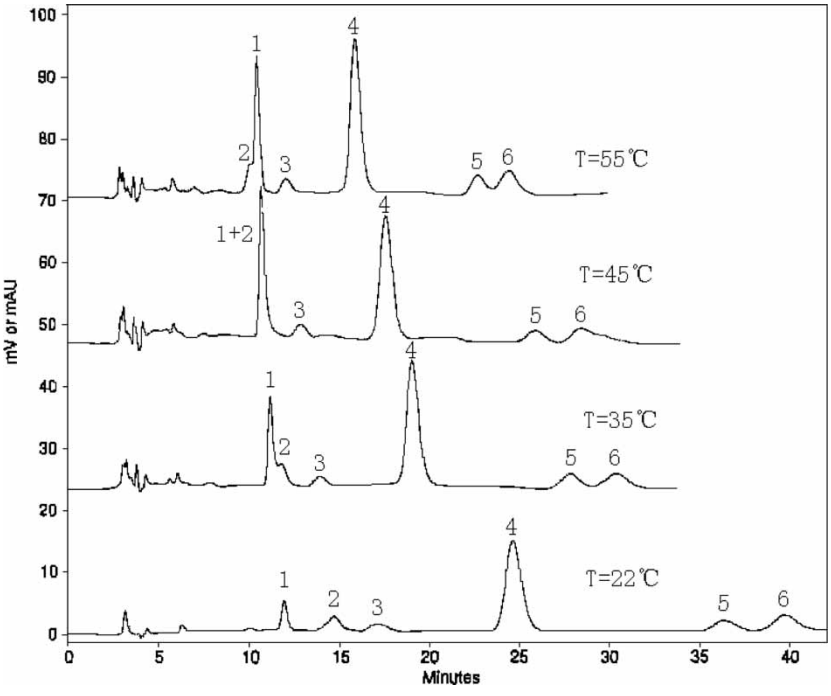

Figure 4. Chromatograms of crude peptide separation at various temperatures. Other conditions are the same as given in EXPERIMENTAL section.

Effect of Flow Rate

With the reduction of flow rate, column efficiencies were slightly improved (Table 2). On the other hand, retention times were prolonged, accompanying increasing peak width and asymmetry. However, the change in peak height was abnormal, among the four flow rates tested, the value of peak height at

Table 2. Effect of flow rate on the retention of peptide

Flow-rate (mL/min)	t_R (min)	α^a	A	H	$W_{1/2}$	As	Plates/m
1.2	20.9	0.75/1.49	722271	12059	0.92	1.09	11346
1.0	24.7	0.69/1.54	877217	13163	0.97	1.09	14096
0.8	33.1	0.66/1.56	1086065	12599	1.32	1.12	14948
0.6	44.8	0.64/1.58	1438360	11837	1.58	1.14	17876

[a]α is separation factor of target peak and its impurities before and after it, respectively.

Other conditions are the same as given in EXPERIMENTAL section.

1.0 mL/min flow-rate was the highest. It indicated that there is an optimum flow rate for the separation. Too fast a flow rate (1.2 mL/min) was unfavorable for the adsorption process, leading to a low peak height. When the flow rate was decreased to 1.0 mL/min, the adsorption achieved equilibrium, thus, the height of the elution peak was increased. However, a further drop in flow rate caused the solute to diffuse, accordingly, a decrease in peak height was found. Therefore, 1.0 mL/min was the optimal choice in this work.

On the base of the study, the ideal separation conditions consisted of: mobile phase THF:water (30/70, v/v) + 0.1%TFA at a flow-rate of 1.0 mL/min and room temperature.

Preparative Separation

Since the goals of the separation process in analytical and preparative chromatography are different, the optimizations of chromatographic conditions are also different between them. In analytical chromatography, we want to generate quantitative information and are interested in a resolution of each peak pair in the sample. Usually, it is conducted under the condition of linear chromatography. In preparative chromatography we want to produce a more pure target compound and focus on how to maximize the separation factor between target component and the closest impurities. The process is normally carried out at the condition of nonlinear chromatography, and column overloads as much as possible. Therefore, the optimum condition for a specific sample in preparative chromatography can't simply scale up from an analytical one.

Optimization of Sample Size

Under the situation of overloading, the profile of the isotherm plays an important role. As shown in Figure 5, with increasing sample size, the retention time of the main peak became shorter and shorter. The peak shapes gradually changed from symmetric to triangular, with a steep front and a diffuse rear. These profiles implied that adsorption isotherm of the target peptide in the elution conditions was Langmuirian. Due to the displacement effect caused by Langmuirian adsorption behavior of the sample, the impurity before the target component was pushed forward by the main peak, thus the overlap band strategy could be used, which could raise the product rate by three times in a large sample.[14] Certainly, because of the displacement effect, some of the impurity after the main peak can chisel in the tail of the major peak. But, the concentration of the target compound was very dilute at the tail, even though an earlier collected point was chosen, which had a slight influence on the final yield. Therefore, the displacement effect can make good use of the capacity of column, providing a more economical operation.

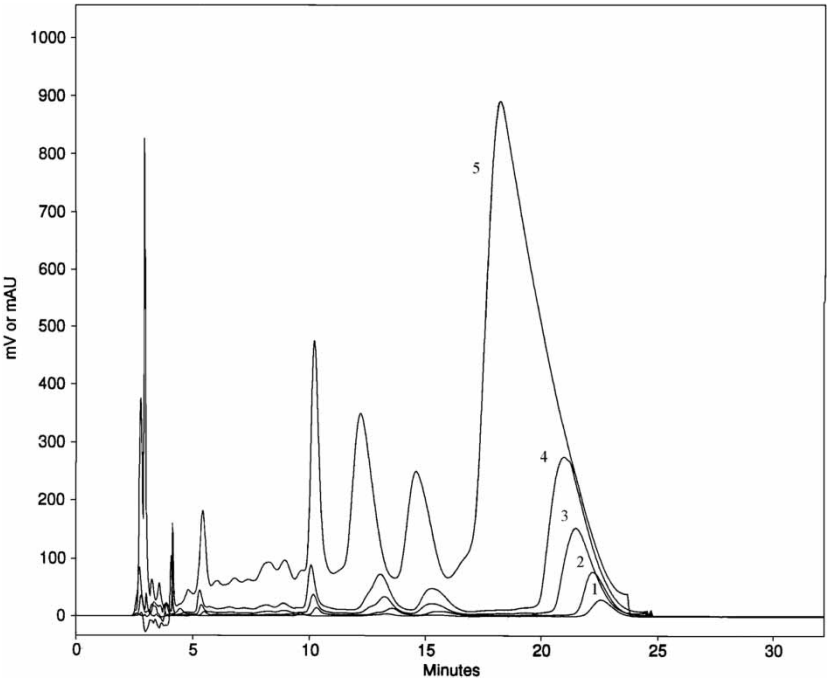

Figure 5. Chromatograms of crude peptide with increasing the sample size. (1) 0.04 mg; (2) 0.09 mg; (3) 0.15 mg; (4) 0.2 mg; (5) 1.0 mg. Other conditions are the same as given in EXPERIMENTAL section.

In order to determine the optimal sample loading, the sample size in the pre run was gradually increased. The data in Table 3 were the analytical results of pooled fractions at a purity above 98%. (According to the pharmaceutical company's criterion, the purity of the final product must be above 98%). With the increase of the injection sample, the percent of the product meeting our desire was decreased, but the absolute amount of the product was increased. When the sample size reached 10 mg, the yield was the greatest in our experiments. So, 10 mg of raw sample was injected per separation unit (Figure 6). Figure 7 presented the analytical chromatogram of pooled fractions collected from point B to C. Compared with the raw sample—50% main component was contained, the purity of the final product was increased to above 98% with recovery 80.5%.

Regeneration Column

In spite of its higher speed and efficiency, the application of preparative chromatography isn't as popular as other traditional chemical isolation

Table 3. The results of fractions analysis with different sample size

Feed load (mg)	[a]The amount of the pure product (mg)	(%)
1	0.49	97.3
2	0.96	96.1
3	1.42	94.5
5	2.29	91.6
8	3.50	87.4
10	4.03	80.5

[a]The product with purity above 98%.

Chromatographic conditions are the same as given in EXPERIMENTAL section.

techniques. The main reason is due to the fact that it is a more expensive method. According to statistics, in many cases, more than 50% of the purification cost is spent on solvents.[15] Generally, the consumption of organic solvent consists of two parts: one is to elute the interesting compound

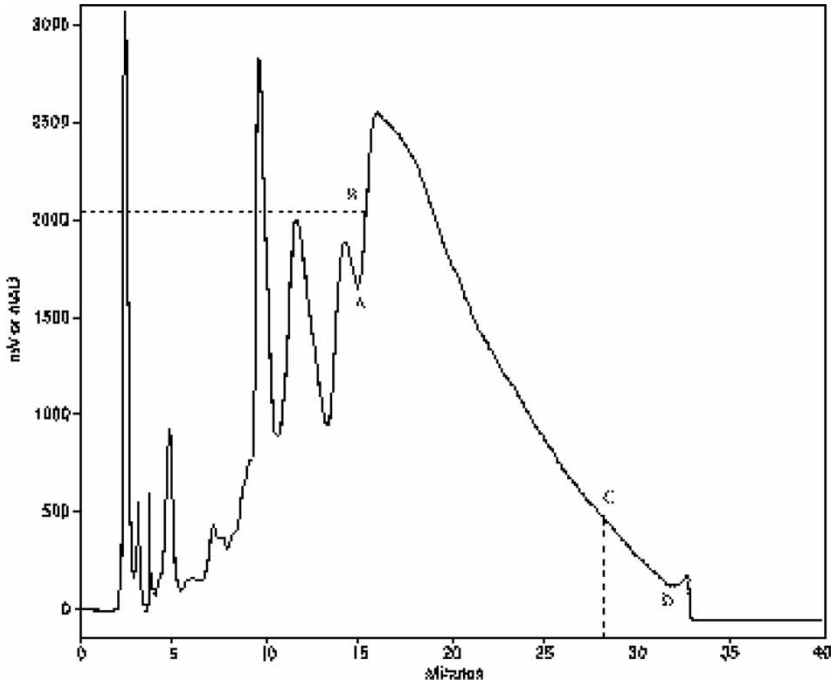

Figure 6. Preparative chromatogram of 10 mg raw sample. Chromatographic conditions as given in EXPERIMENTAL section.

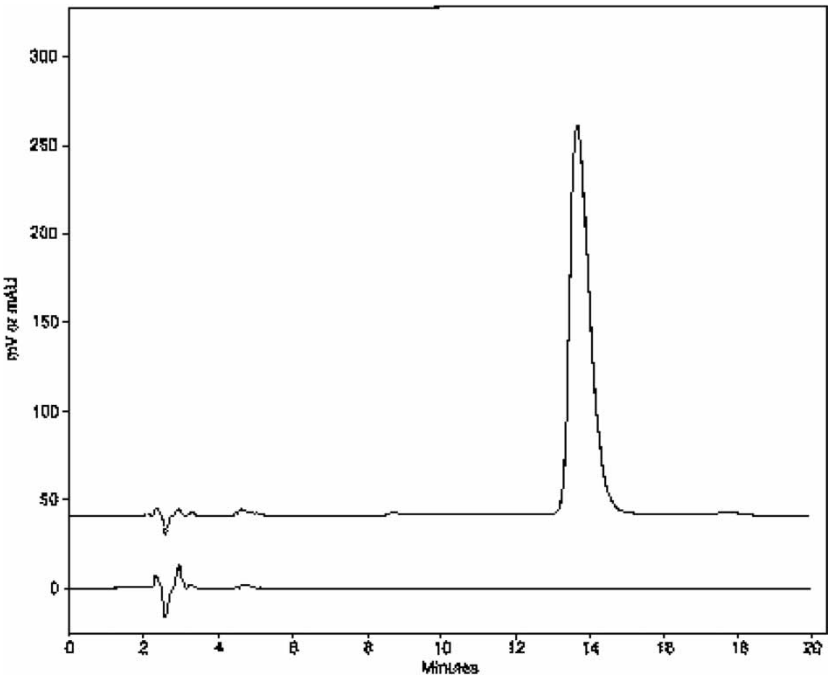

Figure 7. Test for the purity of the collected fractions. Upper chromatogram: 20 μL of fraction; lower chromatogram: 20 μL mobile phase blank. Other conditions as given in EXPERIMENTAL section.

within a reasonable time; the other is to recondition the column to prepare for the next run. For a given separation, the cost in the elution step should be considered basically fixed. Therefore, the only way to minimize the cycle cost is to minimize those of the second part. A common program of reconditioning the column is gradient elution under high concentration organic solvent, then reequilibrating with the initial mobile phase. In this mode, the run time is independent of the time the target peak appears, but is decided by the most strongly bound component and the time of reequilibrating. In the reequilibration step, at least three to five column volumes of solvent are typically required. Moreover, the process of regeneration of the column is more complicated and expensive in gradient elution than isocratic elution.[16] Therefore, these two processes are money and time consuming.

In order to minimize solvent consumption, a backflushing technique was introduced. In most cases, the backflushing technique is used to clean or preconcentrate the sample on-line through column switching, in order to increase sensitivity.[17,18] When it is used for purification of a peptide, the sample was injected to the inlet of the column; once the objective component was collected, the mobile phase was pumped from the outlet by turning the

valve. This operation allows all compounds to disappear along the column rapidly. Theoretically, whether the retention is strong or weak, all retained compounds should leave the column simultaneously in this step. The two processes take the same time under the same mobile phase. Certainly, in a practical run, because the sample diffuses in the column, the cleaning step takes a little longer (shown in Figure 8). With backflushing methods, the cost and time only relied on the nature of the target component. The earlier the main peak emerges, the shorter the run time spends. When the time of the major peak eluted is less than half of the retention time of the strongest resident, the backflushing elution shows an obvious advantage.[19] In our case, as shown in Figure 8, at isocratic elution, the last visible peak is eluted at 114 min, which is about five times of that of the main peak (25 mins). Actually, some other compounds were likely to be left on the column at that time, for the baseline wasn't perfectly flat and the later elution peak was too broad to detect. Therefore, in spite of a long time elution, we can't ensure all of the impurities are removed from the column. However, with the backflushing technique, the total run time would be about 53 mins in a cycle. Compared with the two cases, the introduction of

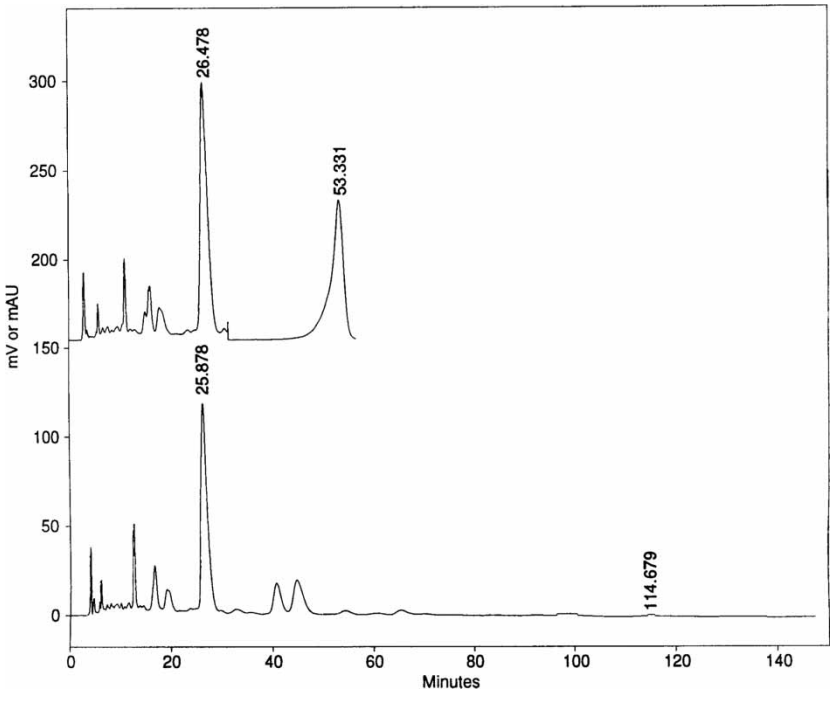

Figure 8. Chromatograms of peptide eluting at different modes. Top line: backflushing program; bottom line: isocratic elution. Other conditions are the same as given in EXPERIMENTAL section.

a backflushing step can not only ensure that each impurity in the crude sample eluted out of column (if the interaction between impurity and stationary phase is reversed), but it also saves time and solvent consumption.

Furthermore, column reequilibration wasn't needed anymore because of the use of the same mobile phase during the cycle run. At the same time, only one pump is needed, thus the cost burden on equipment can be decreased. According to the advantages discussed above, the backflushing mode is an inexpensive but efficient technique.

CONCLUSION

We have described an RPLC method for the purification of peptides from crude products based on THF contained mobile phase and a backflushing process. Experiment data proved THF was an appropriate organic modifier to some peptide samples. The optimum condition for the separation of peptides was THF/water (30:70, v/v) + 0.1% TFA, at a flow rate of 1.0 mL/min and ambient temperature. The purity of the final product was above 98%, with the recovery of 80.5% for a 10 mg raw sample. This method can be used as a guide to promote the more frequent use of THF as organic modifiers in peptide purification, and scale up to industrially preparative separation. Moreover, the application of a backflushing technique is simple, quick, and effective.

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