HCI 프로그램을 이용한 RP-HPLC에서의 펩타이드의 최적 분리 조건

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Optimum separation conditions of peptides in RP-HPLC by HCI program

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Introduction

Optimization in reverse phase-high performance liquid chromatography (RP-HPLC) involves the selection of experimental condition for adequate separation and acceptable retention time for each individually samples. But in chemical and pharmaceutical laboratories obtaining a balance between resolution and analysis time is not always easy. An efficient optimization method should be employed during the method development process in order to deal with these optimization problems. Computers have been used as an aid in high performance liquid chromatography method development since the late 1970s.[1] The method developments by software were frequently demonstrated, and the applications using the software have been increasing. Especially HCI program was originally developed by High-Purity Separation Lab., Inha University for the purpose of the optimization of chromatographic separation. Now the scope of HCI program is limited to analytical condition. It can be utilized in normal-phase as well as reversed-phase liquid chromatography for the isocratic and gradient modes. The basic function enables to predict the retention time of samples in a given mobile phase composition, and additionally column efficiency and resolution, elution profile of sample in specific and optimized operating conditions. In this work, the optimization of mobile phase condition for 4 biological-active peptides was determined by HCI program.

Naturally occurring peptides show a wide variety of biological effects. The main function of bradykinin is to increase the sensation of pain.[2] Bradykinin also sensitizes free nerve endings, making them hypersensitive to heat and light touch and creating an overall sensation of soreness. Leucine enkephalins[3] have been associated with addiction to and withdrawal from morphine. This decrease in enkephalin neural activity necessitates an increase in the amount of morphine in order to maintain the same level of analgesia. Its presence in the blood launches a chain of reactions that result in the production of angiotensin II,²Ⅲ[4] a molecule that raises blood pressure. In addition to its role in raising blood pressure, angiotensin II promotes the overgrowth of cells, called hypertrophy, in the heart and blood vessel walls and in the kidneys. The hypertrophic response to angiotensin II is a major problem leading to heart failure, atherosclerosis, and kidney failure.

RP-HPLC is the most widely used analytical technique for separation and isolation of peptides.[5] So the mobile phase is a mixture of an aqueous and organic solvent in which the hydrophobic interaction between peptides and the non-polar stationary phase allows peptide isolation.[6]

In this paper, HCI program were used to optimize the separation of peptides by

RP-HPLC. A modified equation was suggested for calculating the distances migrated by the solutes in step and gradient mode. The optimum composition of mobile phase for the separation of the 4 peptides was obtained on the basis of resolutions and separation times. The elution profiles in the optimal mobile phase condition and operating mode were calculated by plate theory to compare with experimental data.

Experimental

Reagents

Four standard peptides, AngiotensinⅡ (Asp−Arg−Val−Tyr−Ile−His−Pro−Phe), [Val^4]−AngiotensinⅢ (Arg−Val−Try−Val−His−Pro−Phe), Bradykinin (Arg−Pro−Pro−Gly−Phe−Ser−Pro−Phe−Arg) and [$_D$−Ala^2]−Leucine Enkephalin (Tyr−$_D$−Ala−Gly−Phe−Leu), were purchased from Sigma (St. Louis, MO, USA). HPLC grade solvent, acetonitrile was from Ducksan Pure Chemical (Kyungki−Do, Korea). Trifluoroacetic Acid (TFA) was purchased from Sigma (St. Louis, MO, USA). Water filtered by Milipore ultra pure water system (Milipore, Bedford, MA, USA).

Sample preparation

Four standard peptides, 5mg, were dissolved in water 1ml, then the concentration of the solutions were adjusted to 5000㎍/㎖, respectively. The constant injection volume of mixtures solution, 3l, was used throughout.

Apparatus and method

HPLC was performed using Waters 600S solvent delivery system (Waters, Milford, MA, U.S.A.). 2487 UV dual channel detector was used (Waters, Milford, MA, U.S.A.). Data acquisition system was Millenium^{32} (Waters) installed in HP Vectra 500 PC. Water filtered by Milipore ultra−pure water system (Milipore, Bedford, MA, USA).
The mobile phases were degassed with helium. The flow rate of mobile phase was 1ml/min and monitored at the fixed wavelength of 220nm. The column was purchased from Alltech Co. The column size was 0.4615 cm and packed by C18, 100Å, 5㎛. All the experimental runs were carried out in ambient temperature. The dead volume (V_0) was determined as the retention volume of 20㎛ of acetonitrile.

Results and Discussion

The four peptides were separated by change in mobile phase compositions. Based on the plate theory, elution profiles might be predicted by introducing the concept of solute migration in mobile phase, the linear and the quadratic dependency of ln k in terms of the content of organic modifier. The elution order of four peptides is Angiotensin Ⅲ, Leucine Enkephalin, Bradykinin and Angiotensin Ⅱ. In Fig. 1, the peaks were observed at the mobile phase composition 80/20, 0.1% TFA in water/0.1% TFA in acetonitrile (vol. %). And in Fig. 2, the first mobile phase composition was water with 0.1% TFA/acetonitrile with 0.1% TFA, 80/20 vol.%, then after 7 min to 8 min, the second composition of mobile phase was linear-changed to 79/21 vol.%, and finally after 8 min, it was kept at the isocratic mode. Regressions were performed with Eq. $\ln k = A+BF+CF^2$ to obtain empirical constants of each solute. The results were presented in Table 1. Regression coefficients of four peptides are higher than 0.99. Without HCI program aid many more experiments would have been

necessary and one may probably assume that it would have been very uncertain to attain an equally good separation as that presented in results.

Table 1 Empirical constants and regression coefficients of peptides by a quadratic relationship

Material	$\ln k = A + BF + CF^2$			Regression coefficient
	A	B	C	
Angiotensin	6.700	-0.336	0.0035	0.9989
Leucine Enkephalin	5.851	-0.269	0.0026	0.9983
Bradykinin	7.825	-0.381	0.0039	0.9980
Angiotensin	8.014	-0.384	0.0040	0.9987

Acknowledgements

The authors gratefully acknowledged for the financial support by Center for Advanced Bioseparation Technology.

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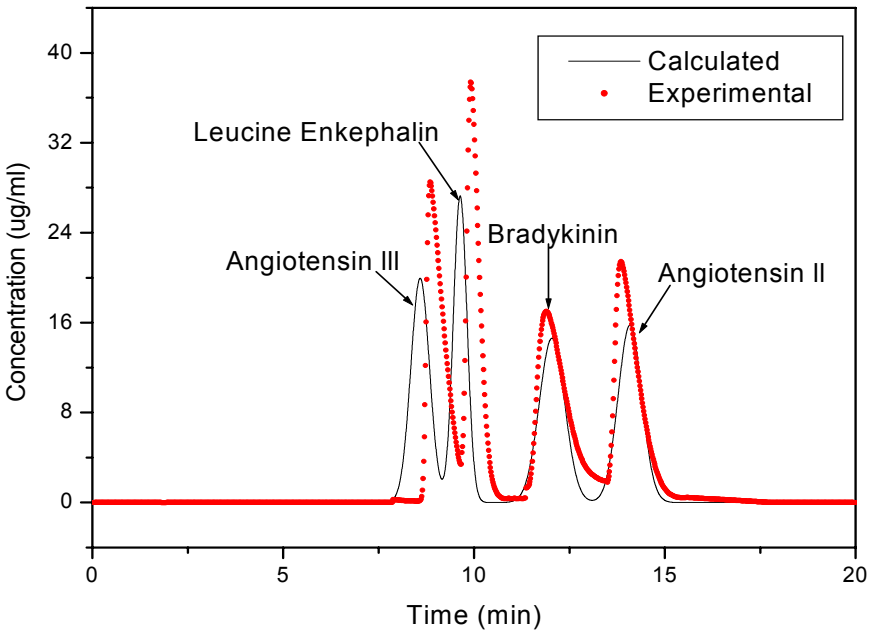

Fig. 1. Comparison of the experimental and calculated profiles in optimum isocratic condition. (F: 20%)

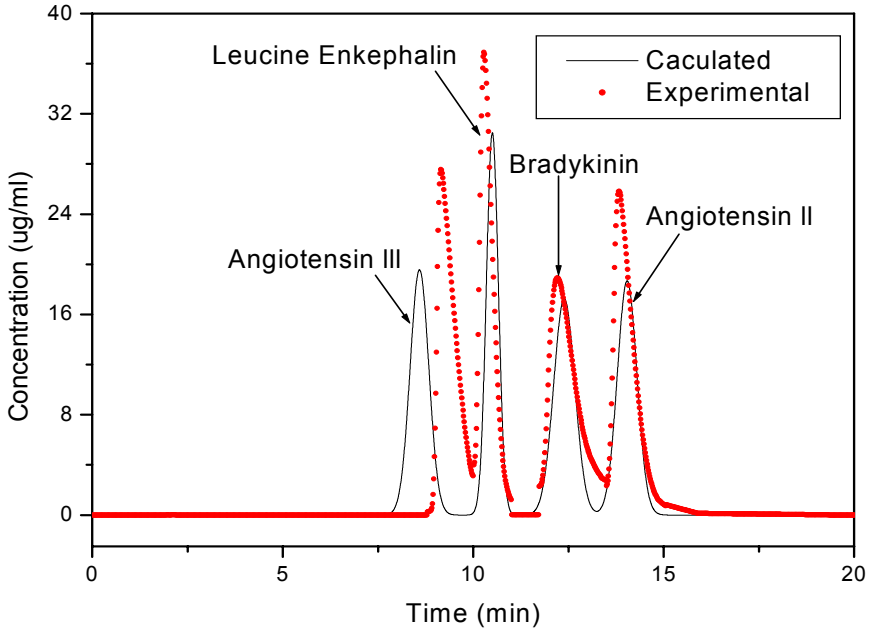

Fig. 2. Comparison of the experimental and calculated profiles in optimum gradient condition (F1: 20%, F2: 21%, $V_{g,1}$: 7 min, $V_{g,\infty}$: 8 min)