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Short communication

Application of phase optimized liquid chromatography to oligopeptide separations

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ABSTRACT

Stationary phase optimized liquid chromatography (POPLC) has been applied to the separation of oligopeptides. The retention factors and theoretical plate numbers of 13 peptides were determined on five different stationary phases. Based on these values, an optimal stationary phase composition of 250 mm total length consisting of 3 segments of 20 mm octadecyl silica, 10 mm phenyl silica and 220 mm embedded polar octadecyl silica was calculated by the optimizer software. Good agreement between the calculated and experimental chromatograms was observed. In order to achieve short analysis time and baseline separation of all peptides gradient elution and optimization of the column temperature were performed. The optimized mobile phase conditions consisted of (A) acetonitrile and (B) 0.025 M aqueous sodium phosphate buffer, pH 3.0, operated at 10% A (0–12 min) followed by 10–50% A (12–32 min) at a flow rate of 0.5 mL min^{-1} and column temperature of 35 °C. Using these conditions all peptides could be separated in less than 30 min with good resolution and peak symmetry.

1. Introduction

Typically, HPLC method development starts with the selection of the stationary phase and subsequent optimization of mobile phase and operating conditions in order to achieve the appropriate separation selectivity. The selection of the stationary phase depends either on the testing of several chromatographic columns or on the experience of the researcher. A "true" optimization of the stationary phase is hardly performed. Recently, the concept of stationary phase optimized selectivity liquid chromatography (SOS-LC) was developed by Nyiredy and Szucs [1–3] which was commercialized under the name POPLC (phase optimized liquid chromatography) [4]. The technique transfers the "PRISMA" model developed for mobile phase optimization in liquid chromatography [5,6] based on the Snyder's solvent classification [7] and solvent strength prism in multisolvent elution [8] to the optimization of the composition of stationary phases. Column segments containing different stationary phases such as cyano, phenyl or octadecyl silica are combined by computer-aided modeling based on experimental behavior with the individual stationary phases under isocratic mobile phase conditions. The retention time, the plate number of each peak and the hold-up time of the column and the system, respectively, are determined and entered into the optimizer software which calculates the combination of the stationary phases. This is subsequently followed by directly connecting the dead volume free column segments at the length suggested by the software. Further modifications of the mobile phase are not necessary in most cases but can be performed if desired.

The POPLC approach has been used for the separation of flavonoids [2], pesticides [2], steroids [9], nitroaromatic explosives [10] and for the determination of related compounds in synthetic thyroid hormones [11], but the technique has not been applied to the analysis of oligopeptides which comprise an important class of pharmacologically active compounds. Thus, the present study was conducted in order to evaluate the potential of POPLC for the separation of small peptides.

2. Experimental

2.1. Chemicals

HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany), uracil was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The peptides were obtained from

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Sigma–Aldrich (Chemie GmbH, Germany) or Bachem AG (Heidelberg, Germany). Water was purified from a Milli-Q water purification system (Millipore AS, Bedford, MA, USA).

2.2. HPLC separation

Chromatography was performed on a Dionex UltiMate 3000 HPLC system with a UV diode array detector set at 215 nm, an autosampler and a column thermostat (Dionex GmbH, Idstein Germany). Operation, data acquisition and analysis were carried out by the Chromeleon software (Dionex Softron GmbH, Germany). The POPLC column system (Bischoff Chromatography, Leonberg, Germany) consisted of pre-packed column cartridges of 3 mm ID of variable length containing octadecyl silica (C18), triacontyl silica (C30), phenyl silica (Phenyl), cyanopropyl silica (CN) and embedded polar octadecyl silica (EPS). The average particle size was 5 μm with a pore size of 100 Å except for C30 (200 Å). The mobile phase consisted of (A) acetonitrile and (B) sodium phosphate buffer (0.025 M, pH 3.0). The gradient elution program was 10% A (0–12 min), 10–50% A (12–32 min) at a flow rate of 0.5 mL min^{-1}. The mixture solution of peptides was prepared in acetonitrile/water (13:87) at a concentration of about 15 μg mL^{-1} for most of peptides except for peptide 13 at a lower concentration. The hold-up volume was determined using a 0.05% (m/v) solution of uracil. The injection volume was 10 μL.

3. Results and discussion

The strategy of stationary phase optimization consists of four main steps: (1) basic measurements, (2) generation of possible combinations by the software, (3) prediction and determination of the optimum combination and (4) testing of the conditions and further mobile phase and operating conditions optimization if necessary [1]. In order to obtain initial mobile conditions a 20 mm C-18 column was selected. According to the manufacturer's guideline [12] a retention factor k between 5 and 15 should be achieved for the last eluted peak. The k value is determined according to:

$$k = \frac{t_R - t_M}{t_M - t_M^{EC}} \quad (1)$$

where t_R is the total retention time of the last eluted analyte, t_M the hold-up time of the column and t_M^{EC} is the hold-up time of the entire system. In a preliminary study, the peptides 1–9 co-eluted. In order to separate these nine polar peptides, the k-value of peptide 10 instead of the last eluted peptide was evaluated based on Eq. (1). Subsequently, a mobile phase of acetonitrile–0.025 M sodium phosphate buffer, pH 3.0 (13:87, v/v), was selected for the basic measurements.

3.1. Basic measurements

The mixture of the 13 peptides comprised analytes of varying chain length and amino acid composition including peptide diastereomers (Ala-D-PheOH and D-Ala-PheOH) and isomeric aspartyl peptides (Asp-PheOMe and β-Asp-PheOMe). The mixture was separated on 120 mm columns of the individual stationary phases, i.e. cyanopropyl silica (CN), phenyl silica (Ph), octadecyl silica (C18), triacontyl silica (C30) and embedded polar octadecyl silica (EPS), employing isocratic elution with acetonitrile–0.025 M sodium phosphate buffer, pH 3.0 (13:87, v/v) as mobile phase. The results are illustrated in Fig. 1. Identification of the peptides was performed by comparison of the retention times injecting the individual compounds. Due to different interaction mechanisms between the stationary phases and the peptides, the five kinds of columns showed different separation selectivity for the peptide analytes. The lowest retention for all peptide analytes was observed with the cyanopropyl silica phase, followed by the phenyl silica phase. Peptides 10, 11 and 12 were readily separated from the other peptides.

3.2. Stationary phase optimization

As mentioned above, it is crucial to separate the polar nine co-eluting peptides 1–9. Thus, only the retention times and theoretical plate numbers for the peptides from 1 to 10, as well as the hold-up times of the columns and the hold-up time of

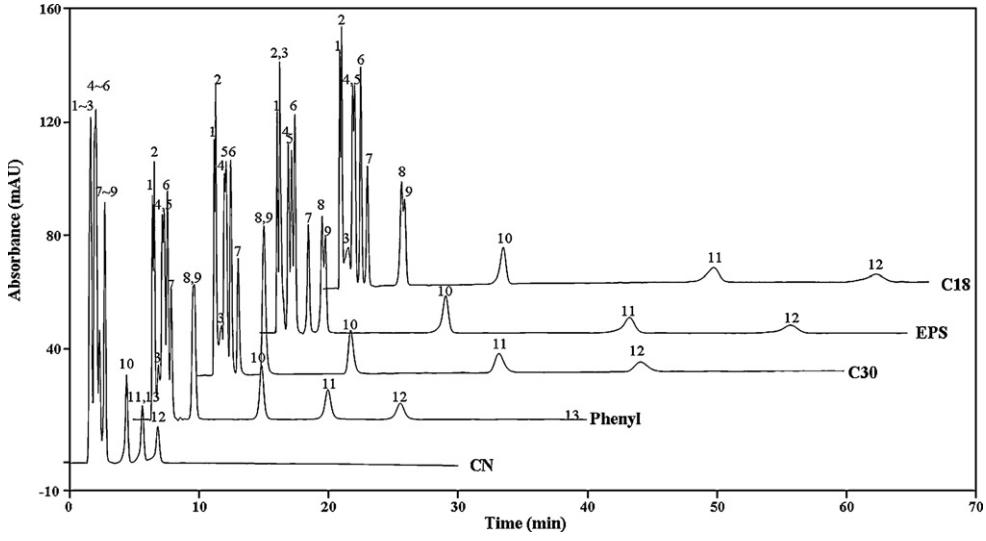

Fig. 1. Isocratic separation of the mixture of 13 peptides on 5 different stationary phases. Columns: 100 mm × 3 mm cyanopropyl silica (CN), 120 mm × 3 mm phenyl silica (Phenyl), triacontyl silica (C30), 100 mm × 3 mm embedded polar octadecyl silica (EPS) and 100 mm × 3 mm octadecyl silica (C18); mobile phase: acetonitrile–sodium phosphate buffer (0.025 M, pH 3.0) (13:87, v/v); flow rate: 0.5 mL min^{-1}; temperature: 30 °C; injection volume: 10 μl; UV detection performed at 215 nm. For peak identification see Table 1.

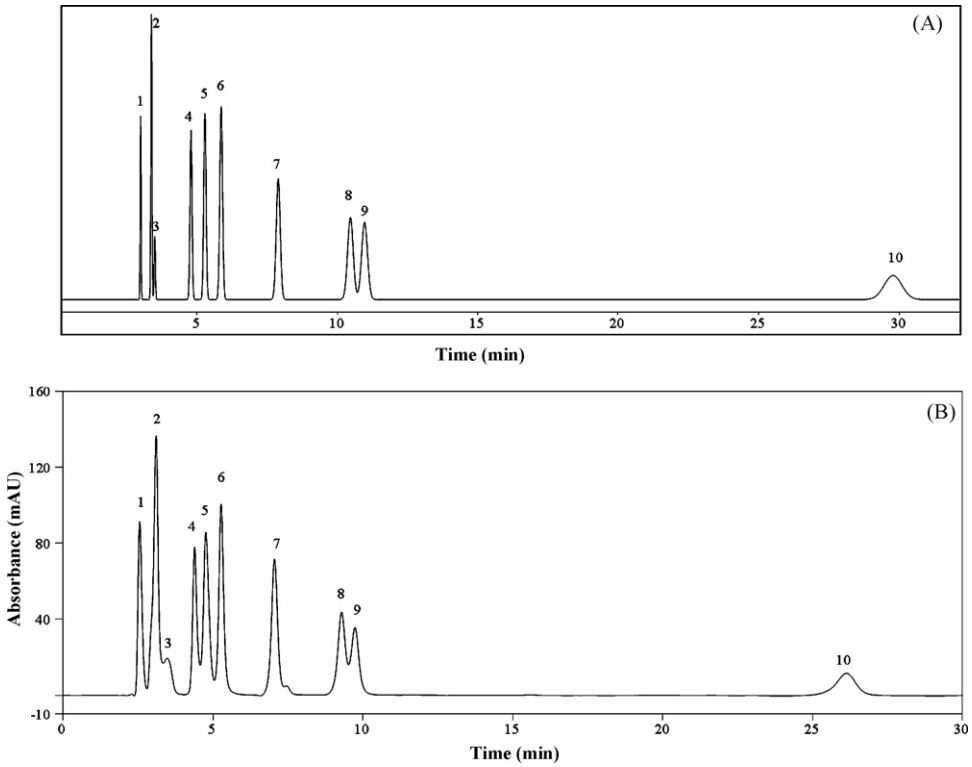

Fig. 2. Comparison between (A) predicted chromatogram and (B) experimental chromatogram of 10 peptide analytes under isocratic conditions. Experimental conditions: Column: 250 mm × 3.0 mm i.d. (20 mm C18 + 10 mm Phenyl + 220 mm EPS); mobile phase: acetonitrile–sodium phosphate buffer (0.025 M, pH 3.0) (13:87, v/v); flow rate: 0.5 mL min^{-1}; temperature: 30 °C; injection volume: 10 μl; UV detection performed at 215 nm. For peak identification see Table 1.

the system, were entered into the optimizer software. Further parameters entered to calculate the best column composition were a maximal analysis time of 30 min, a minimum resolution of 1.2 and a maximum column length of 250 mm. The suggested result was a 250 mm column consisting of 3 segments of 20 mm octadecyl silica, 10 mm phenyl silica and 220 mm embedded polar octadecyl silica. A comparison between the predicted chromatogram and an experimental chromatogram using isocratic elution (acetonitrile–0.025 M sodium phosphate buffer, pH 3.0, 13:87, v/v) is shown in Fig. 2. Both chromatograms are in good agreement. Peptide 10 eluted around 30 min which suggested that the peptides 11–13 would elute much later under the isocratic mobile phase condition. Moreover, not all analytes were baseline separated. For example, low resolution between peptides 2 and 3, and the isomeric aspartyl peptides 8 and 9 was observed. The order of the individual segments of the column did not affect the separation.

3.3. Further optimization of mobile phase and operating conditions

A linear gradient increasing the acetonitrile content from 10% to 50% within 20 min was developed following an initial isocratic elution for the first 12 min using a mobile phase of acetonitrile–0.025 M sodium phosphate buffer, pH 3.0 (10:90, v/v). Further optimization of column temperature to 35 °C led to the optimized separation conditions shown in Fig. 3. The chromatographic parameters of the peaks are summarized in Table 1.

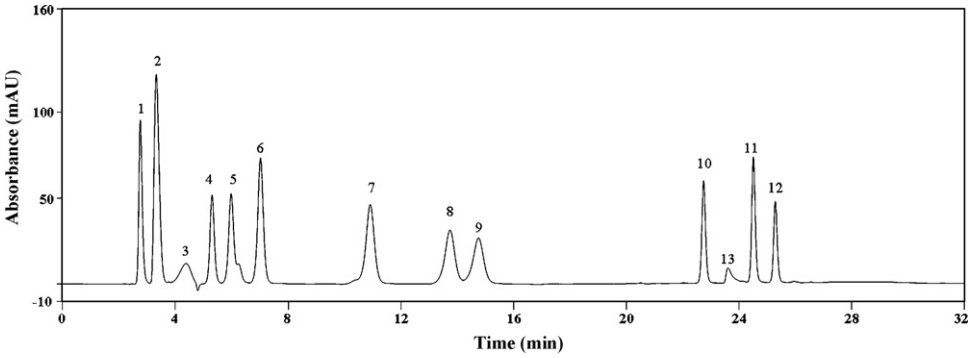

Fig. 3. Chromatogram of the separation of the peptide analytes with optimized gradient elution. Experimental conditions: Column: 250 mm × 3.0 mm i.d. (20 mm C18 + 10 mm phenyl + 220 mm EPS); mobile phase: acetonitrile–0.025 M potassium phosphate buffer (pH 3.0) (10:90, v/v) for 12 min, increasing acetonitrile to 50% in 32 min; temperature: 35 °C; flow rate: 0.5 mL min^{-1}; UV detection performed at 215 nm. For peak identification see Table 1.

Table 1
Chromatographic parameters of the peptides under optimized separation conditions.

No.	Peptide	Retention time [min]	Resolution	Symmetry factor	Plate number[a]
1	Tyr-Gly-GlyOH	2.76	–	1.24	2539
2	Gly-Gly-TyrOH	3.33	2.04	1.28	1560
3	Leu-Gly-ProOH	4.38	2.03	0.86	611
4	Ala-PheOH	5.30	1.86	1.09	5798
5	Asp-PheOH	5.98	2.25	1.47	5261
6	Ala-D-PheOH	7.03	3.03	1.00	6151
7	Gly-Leu-TyrOH	10.92	8.73	0.91	6640
8	Asp-PheOMe	13.74	4.88	0.98	7753
9	β-Asp-PheOMe	14.76	1.59	0.88	7954
10	Met-enkephalin (Tyr-Gly-Gly-Phe-MetOH)	22.74	17.77	1.11	144801
13	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-LeuOH)	23.61	2.95	1.98	72880
11	Leu-enkephalin (Tyr-Gly-Gly-Phe-LeuOH)	24.52	3.14	1.08	182711
12	D-Ala[2]-Leu-enkephalin (Tyr-D-Ala-Gly-Phe-LeuOH)	25.29	3.39	1.11	194445

[a] Normalized to 1 m of column length.

Satisfactory resolution of at least 1.5 could be achieved for all analytes with a significant reduction of separation time within 30 min. Only few peaks show moderate tailing or fronting, the highest peak symmetry factor was found for angiotensin I (peptide 13) with a value of about 2.

4. Conclusions

Phase optimized liquid chromatography (POPLC) has been applied to the separation of a mixture of oligopeptides for the first time. Following the initial protocol of stationary phase optimization employing an isocratic mobile phase all peptides could be separated in less than 30 min upon further optimization of the mobile phase including linear gradient elution and column temperature. Usually isocratic elution of the analytes is recommended in POPLC method development. However, the current study shows that a linear gradient elution, instead of step gradient elution [11] can also be successfully applied in POPLC application. To the best of our knowledge, this is the first report on the application of gradient elution using POPLC for the separation of oligopeptides.

Acknowledgments

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