On the Purification of Peptides with Size Exclusion Chromatography

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INTRODUCTION

In size exclusion chromatography (SEC) it is often observed that peptides deviate from linear K_{av}-log M_r behaviour (1,2,3). Such deviations may be caused by a combination of adsorption effects and variations in shape. Adsorption may be reduced by changing the mobile phase composition.

Generally, peptides are stable to harsh conditions such as high concentrations of organic solvents, and extreme pH values can be applied as long as they are within the stability range of the chromatography medium.

A study was done to demonstrate the effect of pH and acetonitrile on the retention of five peptides with different properties on Superdex® Peptide, a SEC medium with very high chemical resistance (4). The properties of the peptides differ with respect to charge and hydrophobicity.

A fractional factorial experimental design was used to investigate how the retention volumes of the peptides were affected by changes in pH and acetonitrile concentration. Four replicate runs were made near the "midpoint" (pH 7.2 and concentration of acetonitrile at 15 %) in order to determine the experimental error.

The peptides chosen were gastrin I, somatostatin, pentaglycine, hGHRH (1-29) fragment and P0711 which were investigated at pH 2.1 and 10, with acetonitrile concentrations at 5-30 %.

CONDITIONS

System:	FPLC® System
Detection:	UV 214 nm (5 mm path length)
Column:	Superdex® Peptide HR 10/30 prepacked column (300 x 10 mm I.D.)
Evaluation Software:	FPLCdirector®
Modelling:	MODDE 3.0, Umetri AB, Sweden
Mobile phases:	50 mM phosphate buffer with 0.05 or 0.25 M sodium chloride, addition of acetonitrile to 5, 15 or 30 % at pH 2.1, 7.2 or 10
Sample volume:	25 µl
Sample concentration:	0.2 mg/mL

Table I: Investigated peptides

Peptide	M_r	amino acid sequence
gastrin I	2 126	pGlu-Arg-(Pro)$_2$-Met-(Glu)$_2$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH$_2$
somatostatin SRIF (Growth hormone release inhibiting factor)	1 638	Ala-Gly-Cys-Lys-Asn-(Phe)$_2$-Trp-Lys-Thr-Phe-Thr-Ser-Cys (disulfide bridge 3-14)
pentaglycine	303	Gly-Gly-Gly-Gly-Gly
hGHRH fragment (1-29) NH$_2$/Sermorelin	3 358	Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-(Leu)$_2$-Gln-Asp-Ile-Met-Ser-Arg-NH$_2$
PP0711	2 432	Lys-Thr-Ile-Leu-Lys-Ala-Leu-Gly-Pro-(Ala)$_3$-Thr-Leu-(Glu)$_3$-(Met)$_2$-Thr-Ala-Cys-Gln-Gly-Val-Gly

Chromatographic method

Column preservative (20 % ethanol) was removed by washing with 2 column volumes of Milli-Q water followed by equilibration with mobile phase for 2 column volumes. Gastrin I, hGHRH (1-29), P0711 and pentaglycine were dissolved in Milli-Q* water to a concentration of 0.2 mg/mL. Somatostatin was dissolved in 0.1 % TFA to the same concentration. The mobile phases were vacuum degassed before use.

The peptides were manually injected one by one and eluted isocratically at a flow rate of 1.0 mL/min (76 cm/h) for 50 minutes (about 2 column volumes). The retention volumes were calculated at peak maximum.

* Milli-Q is a registered trademark from Millipore

Experimental design

Fractional factorial design was used to find out how the total variation in response (retention volume) was related to pH and concentration of acetonitrile. Factorial experimental design makes it possible to find out which (if any) factor(s) have a real influence on retention and to find these factor(s) with a minimum of experiments. Four replicate runs were made near the midpoint in order to determinate the experimental error.

Table II: Fractional factorial design, factors

Exp. No.	pH	Conc. of CH_3CN (%)
1	2.1	30
2	10	30
3	2.1	5
4	10	5
0-1	7.2	15
0-2	7.2	15
0-3	7.2	15
0-4	7.2	15

RESULTS AND DISCUSSION

Neutral peptide: pentamer of glycine

The effect of the choice of mobile phase is not so pronounced for the pentamer of glycine. The response surface is consequently almost flat as shown in figure 1B, but some effects are visible in the chromatogram, figure 1A. A hydrophilic environment is most suitable to obtain unretarded elution of the peptide.

Effect of pH
A small effect of pH can be seen.

Effect of acetonitrile
Increasing the content of acetonitrile from 5 to 30 % leads to an increase in the retention volume of the neutral peptide from 16.9 to 18.7 mL.

Acidic peptide: gastrin I

Effect of pH
At pH 2.1, all carboxyl residues are protonated and 30 % acetonitrile in the mobile phase is necessary to give a narrow peak that elutes unretarded (Exp. No. 1). When the content of acetonitrile is reduced to 5 %, the peptide interacts strongly with the matrix and elutes as several small broad peaks with the main peak after more than one column volume (Exp. No. 3). See figure 2A.

At pH 10.0, the deprotonated carboxyl groups of Glu (x6) and Asp (x1) makes gastrin I highly negatively charged and the retention volume of the peptide is not affected by acetonitrile (Exp. No. 2 and 4). This indicates that separation of acidic peptides can be performed without organic solvents at basic pH. Such separations are not possible with silica based matrices, due to their limited chemical stability at high pH.

Effect of acetonitrile
Figure 2B shows the contour plot of the relation between retention volume, and pH and concentration acetonitrile when gastrin I is eluted on Superdex Peptide. If a low pH is chosen the importance of organic solvent is increased. At low concentrations of organic solvent gastrin is significantly retarded. The dominant process governing peptide retention is size exclusion, when chromatography is performed at high pH. The peptide is negatively charged and no matrix interactions can be observed.

Basic peptide: hGHRH fragment (1-29)

The retention volume is only slightly affected of the choice of mobile phase. The response surface is consequently almost flat, as shown in figure 3B, but the chromatogram, figure 3A, shows a difference in peak shape under different conditions.

Effect of pH
The effect of pH on the elution of the basic peptide, hGHRH fragment, is not so obvious as for the acidic peptide gastrin I. The peptide is larger and is also somewhat hydrophobic.

At high pH (10.0), most of the amino residues are deprotonated and the peptide elute in peaks with increased leading in combination of low acetonitrile contents (5 %) (Exp. No. 4). At neutral pH and 15 % acetonitrile the peptide behaves similar as at pH 10.0 (Exp. No. 0). At low pH is the peptide ionised and gives best peak shape both with addition of 30 % acetonitrile and at 5 % acetonitrile (Exp. No. 1 and 3).

Effect of acetonitrile
The best separation of the peptide is performed at 30 % acetonitrile at low pH (Exp. No. 1). But also at pH 10.0 is the separation good if 30 % acetontrile is added (Exp. No. 2).

Hydrophobic peptide with aromatic amino acids: Somatostatin

Effect of pH
The effect of pH is small, which is clearly illustrated in the contour plot (figure 4B) and experiment 1 and 2 in figure 4A.

Effect of acetonitrile
The most important factor for this hydrophobic peptide is the concentration of acetonitrile in the eluent, which is demonstrated in figure 4A and B. Unretarded narrow peaks (13.9 and 14.1 mL) are obtained at 30 % acetonitrile independent of pH (Exp. No. 1 and 2). When the acetonitrile concentration is reduced to 5 %, the retention of somatostatin is significantly increased to 19.0 and 21.9 mL (Exp. No. 3 and 4). 15 % acetonitrile is not enough to suppress the hydrophobic interaction (Exp. 0).

Hydrophobic peptide without aromatic amino acids: PP0711

PP0711 contains a free cysteine residue and dimerises easily. Experiment with 2-Mercapto-ethanol gave one peak (not shown).

The effects of adding acetonitrile or change pH in the mobile phases is not so obvious as for the hydrophobic peptide somatostatin. The peptide is larger and is also somewhat polar. The response surface is consequently almost flat, as shown in figure 5B. The chromatograms (figure 5A) show the dimerisation of the peptide under all conditions.

Table III: Retention volumes for the different peptides in different mobile phases (see table II)

Exp. No.	Pentaglycine (mL)	Gastrin I (mL)	Somatostatin (mL)	hGHRH (mL)	P07011 (mL)
1	18.7	12.6	13.9	11.0	11.2
2	17.9	13.3	14.1	11.8	11.0
3	17.6	32.0	19.0	11.5	11.3
4	16.9	12.4	21.9	12.5	11.0
0-1	18.2	14.1	18.0	12.3	11.4
0-2	18.2	14.0	17.8	12.4	11.5
0-3	18.2	13.8	17.9	12.3	11.4
0-4	18.1	13.8	17.8	12.4	11.4

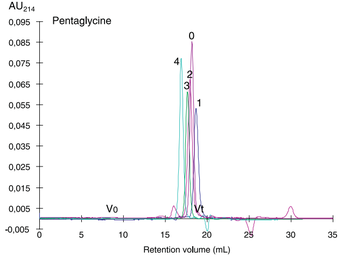

Figure 1A: *Elution profiles of pentaglycine. (0) pH 7.2 and 15 % CH_3CN, (1) pH 2.1 and 30 % CH_3CN, (2) pH 10.0 and 30 % CH_3CN, (3) pH 2.1 and 5 % CH_3CN and (4) pH 10.0 and 5 % CH_3CN.*

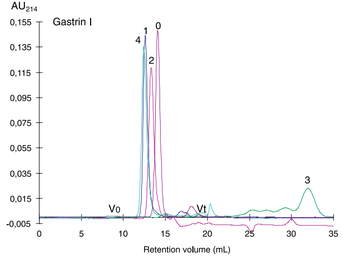

Figure 2A: *Elution profiles of gastrin I. (0) pH 7.2 and 15 % CH_3CN, (1) pH 2.1 and 30 % CH_3CN, (2) pH 10.0 and 30 % CH_3CN, (3) pH 2.1 and 5 % CH_3CN and (4) pH 10.0 and 5 % CH_3CN.*

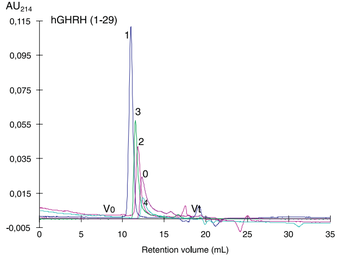

Figure 3A: *Elution profiles of hGHRH fragment (1-29). (0) pH 7.2 and 15 % CH_3CN, (1) pH 2.1 and 30 % CH_3CN, (2) pH 10.0 and 30 % CH_3CN, (3) pH 2.1 and 5 % CH_3CN and (4) pH 10.0 and 5 % CH_3CN.*

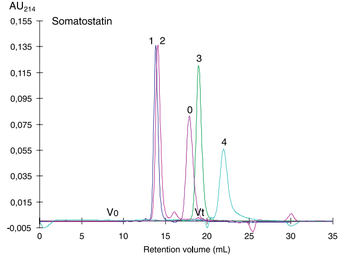

Figure 4A: *Elution profiles of somatostatin. (0) pH 7.2 and 15 % CH_3CN, (1) pH 2.1 and 30 % CH_3CN, (2) pH 10.0 and 30 % CH_3CN, (3) pH 2.1 and 5 % CH_3CN and (4) pH 10.0 and 5 % CH_3CN.*

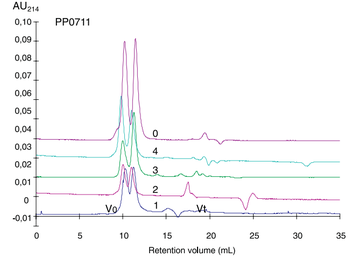

Figure 5A: *Elution profiles of PP0711. (0) pH 7.2 and 15 % CH_3CN, (1) pH 2.1 and 30 % CH_3CN, (2) pH 10.0 and 30 % CH_3CN, (3) pH 2.1 and 5 % CH_3CN and (4) pH 10.0 and 5 % CH_3CN.*

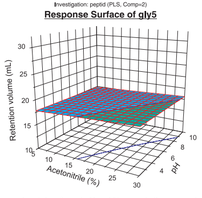

Figure 1B: *Contour plot of the effect of pH and % acetonitrile on retention volume of pentaglycine.*

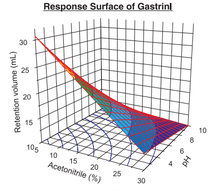

Figure 2B: *Contour plot of the effect of pH and % acetonitrile on retention volume of gastrin I.*

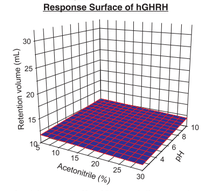

Figure 3B: *Contour plot of the effect of pH and % acetonitrile on retention volume of hGHRH fragment (1-29).*

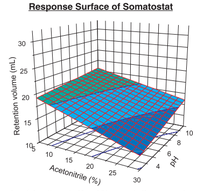

Figure 4B: *Contour plot of the effect of pH and % acetonitrile on retention volume of somatostatin.*

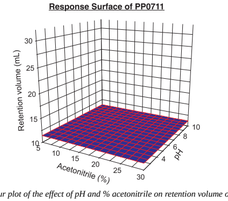

Figure 5B: *Contour plot of the effect of pH and % acetonitrile on retention volume of PP0711.*

CONCLUSION

- Optimisation of the mobile phase facilitate separation of peptides by chromatography on Superdex Peptide.
- Retention of neutral peptides is only slightly affected by pH and hydrophobic modifies.
- Acidic peptides are preferably run at high pH if hydrophilic conditions are required. Elution at low pH requires the addition of 30 % CH_3CN to suppress hydrophobic interactions.
- SEC of hydrophobic peptides requires solvent (ex acetonitrile) in the mobile phase.
- For larger peptides retention volumes are relatively independent of mobile phase composition. Optimisations of the mobile phase composition can be necessary to optimise the peak shape.

References

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