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Short Communication

Determination of the Thermodynamic Behavior of a Therapeutic Peptide in Overloading Conditions in Gradient Elution Chromatography

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ABSTRACT

The aim of this work is to model the adsorption behavior of a cyclic octapeptide, octreotide, in reversed-phase gradient elution liquid chromatography. Adsorption isotherms of the peptide were firstly determined in isocratic conditions, using different peptide concentrations and compositions of the mobile phase (mixtures with different percentage of organic modifier, ranging from 23 to 28% v/v). Inverse Method (IM) was applied to determine the parameters describing the isotherm equation for every composition of mobile phase considered. Then, the isotherm parameters have been correlated to the amount of organic modifier in the mobile phase, through the Linear Solvent Strength model (LSS). In the end, it was possible to predict the chromatographic behavior of the cyclic octapeptide in overloading gradient conditions, the knowledge of which can be useful when scaling the method in preparative conditions.

Keywords: Peptide; Inverse method; Adsorption isotherm; Langmuir isotherm; Nonlinear chromatography

INTRODUCTION

Peptides are a particular class of biomolecules largely employed in pharmaceuticals, nutraceuticals and cosmetics. Not only do they act specifically towards a particular target receptor, making them effective also at very low concentration, but also, they do not accumulate in the human body, and this contributes to avoid dangerous side effects [1-4]. Usually, peptides can be produced through recombinant synthesis or through Liquid-phase or Solid-Phase synthesis [5,6]. Anyway, none of the production methods leads to the single target product, but rather to a wide range of impurities. As a consequence, one or more purification steps are required to reach the purity requirements imposed by regulatory agencies [4]. The main technique used for the purification of peptides is liquid chromatography in preparative conditions, which means that large volumes of feed with high concentration are processed in a single run. Since the amount of product injected into the column in preparative chromatography can be very large, the adsorption isotherm of the compound is nonlinear. Preparative conditions also imply that the retention of analytes is concentration-dependent and moreover chromatographic peaks are not gaussian but show a strong asymmetry such as fronting or tailing. Separation problems related to complex mixtures in overloading conditions are challenging also because the amount of a component adsorbed on the stationary phase depends on the concentration of all the other species in solution.

Usually the operating conditions of the purification process are determined using trial and error strategies, with consequent waste of product and time. The knowledge of thermodynamic equilibrium of the target peptide can give information on the maximum loading and on the affinity of the product for the stationary phase. This can be a help in the design of the separation process, also for large-scale purification and for processes employing continuous chromatography technology [7-12].

The adsorption isotherm of a compound is traditionally determined using Frontal Analysis, a technique which employs large amounts of product. The technique used in this study,

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called Inverse Method, permits to obtain the isotherm parameters injecting only low amounts of the compound of interest, allowing saving material and time [13-16]. The procedure followed to apply the Inverse Method is reported in bibliography [17-20] and it has been described in detail by the authors in a previous paper [21].

In this work, Inverse Method has been applied to obtain the adsorption isotherm of octreotide, a cyclic octapeptide, at different isocratic conditions in reversed-phase chromatography. The correlation of isotherm parameters with the composition of the mobile phase, determined through Linear Solvent Strength (LSS) model, has been used to predict the adsorption behavior of the peptide under overloading and gradient conditions. The prediction of the peak profile in gradient conditions could be exploited during the design of the purification process in preparative conditions.

MATERIALS AND METHODS

The adsorption was studied on a commercial column, a 150 × 4.6 mm Zorbax SB-C18 column, with 5 µm particle size e 80 Å pore size. The synthetic crude of octreotide was obtained by means of Solid Phase Synthesis by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy), which also provided the pure octreotide to be used as a standard.

The measurements were performed using an Agilent 1100 Series Capillary LC system equipped with a photodiode array detector, the wavelength of which was set at 280 nm. Different loops have been used for the calibration of the detector (500 µL) and for the measurements (5, 10, 20 µL).

Solutions with different octreotide concentrations (0.1, 0.3, 0.6, 1.2, 2.0, 4.0 and 6.0 g/L) were prepared to study the overloaded band profiles of the compound of interest both in isocratic and in gradient conditions. The mobile phases used were 0.02% trifluoroacetic acid (TFA) in water (MP-A) and in acetonitrile (MP-B), respectively. The isocratic conditions examined were in a range from 23 to 28% (v/v) of MP-B.

Adsorption isotherms were determined through Inverse Method, which allows obtaining thermodynamic information in few steps using very low amounts of compound. The procedure followed for the Inverse Method and other experimental conditions employed are reported [21].

RESULTS AND DISCUSSION

Firstly, a gradient method has been performed on a solution of crude octreotide to find a range of ϕ (fraction of organic modifier) where the peptide elutes. Taking into account the dwell volume of the system, it has been estimated that the peptide elutes around ϕ=0.25. As a consequence, a range of ϕ between 0.23 and 0.28 has been considered for the isotherm determination.

At infinite dilution, it is possible to affirm that the retention factor is dramatically affected by the amount of organic modifier in the mobile phase, as it can be seen from Figure 1. A variation in the mobile phase from ϕ=0.23 to ϕ=0.28 causes a 5 times reduction in retention factor (from 4.7 to 0.95).

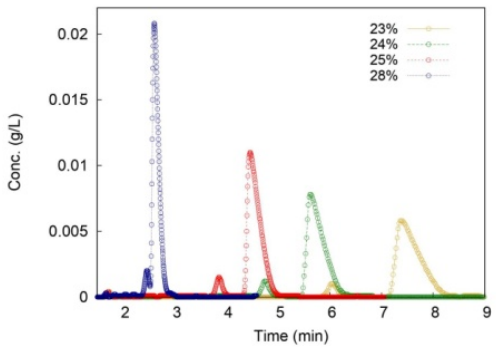

Figure 1: Variation of the retention with the fraction % (v/v) of organic modifier. Comparison between experimental peak profiles of a crude peptide sample at different ϕ, from 0.23 to 0.28. Sample conc=0.6 g/L; Vinj=5 µ L. Reproduced with permissions from study by De Luca et al. [21].

Inverse Method has been applied to determine the adsorption isotherm parameters at each composition of mobile phase considered, for both the crude and the pure octreotide samples. Langmuir, Bilangmuir and Tòth isotherm equations have been tested, but only the Langmuir isotherm appropriately fitted experimental peak profiles. The Langmuir isotherm equation is written as (Equation 1):

$$q = \frac{q_s b C}{1 + bC} \qquad (1)$$

where q_s is the adsorption saturation capacity and b is the adsorption equilibrium constant; the product $q_s \times b$ is the Henry constant (a). By changing the mobile phase composition, the adsorption isotherm model does not change, but its parameters q_s and b do. Anyway, if the range of concentrations of organic modifier is narrow, q_s is likely to be constant; therefore, only b changes during the gradient, which means that b is a function of ϕ. The reader is addressed to De Luca et al. [21] for a theoretical discussion of the formulas employed.

To make a comparison between the peak profiles of different crude peptide concentrations at a given composition of mobile phase Figure 2 is reported, which shows both the experimental and the simulated overloaded peaks. The equilibrium-dispersive model, which is the model chosen in this study for the calculations of the Inverse Method, neglects kinetic phenomena which possibly occur in fact. This could be the reason for small differences between the measured and the experimental peak profiles, especially in the front part and for higher concentrations. On the other side, the agreement in the rear part is perfect at every concentration. The agreement between experimental and theoretical peak profiles has been found to be excellent also for the pure octreotide samples.

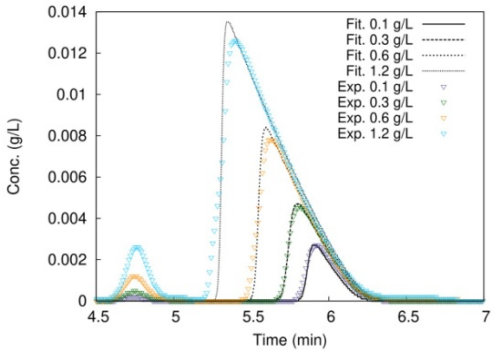

Figure 2: Agreement between calculated and experimental profiles at four different peptide concentrations in isocratic conditions. The black lines are the theoretical profiles obtained through Inverse Method using a Langmuir isotherm model, whereas the colored circles represent the experimental chromatograms. Vinj=5 µL; **ϕ**=0.24. Reproduced with permissions from [21].

The Inverse Method calculations give back a value for q_s and b for every isocratic condition; q_s and b do not change by varying the peptide concentration at a given **ϕ**. From Table 1 it can be noted that, as expected, in the narrow range of **ϕ** considered q_s values are very similar, around 0.69 g/L. On the contrary, b varies with **ϕ** following an exponential trend:

$$b(\phi) = b_0 e^{(-S\phi)} \qquad (2)$$

Where the parameter S is a coefficient characteristic of the system solute-mobile phase and b_0 is the adsorption constant extrapolated at **ϕ**=0. These parameters are found to be 29 and 6.3×10^3 L/g respectively; they are employed to predict the overloaded peak profiles when the elution takes place not in isocratic but in gradient conditions. The Langmuir isotherm is modified to keep into account that b is not constant anymore during the elution, but it changes while changing the amount of organic modifier during the gradient, as described in Equation 3:

$$q = \frac{q_s C b_0 e^{(-S\phi)}}{1 + C b_0 e^{(-S\phi)}} \qquad (3)$$

The equilibrium-dispersive model can be solved using the Langmuir isotherm modified (Equation 3) and the values of q_s, b_0 and S previously found. The outcome of the calculations is a simulated peak profile in gradient conditions, which is to be compared to the experimental peak also obtained in gradient conditions.

As it can be noted from Figure 3, the agreement between the theoretical profiles and the experimental peak is very good at each concentration. In the case of Figure 3, the injection volume is only 5 µL; other measurements and calculations with higher injection volumes (10, 20 µL) have been performed to test the reliability of the model.

It was found that even at higher loading, the match between the predicted and the experimental peaks is still very satisfactory. Thus, the model developed permits to predict the thermodynamic behavior of the peptide in overloading gradient conditions using only small amounts of product, in the order of µg.

Table 1: Parameters obtained at different **ϕ** by fitting the experimental peak profiles using Inverse Method with a Langmuir isotherm model.

ϕ	a	b (L/g)	q_s (g/L)
0.23	5.86	8.14	0.72
0.24	4.21	5.69	0.74
0.25	3.07	4.65	0.66
0.28	1.17	1.86	0.63

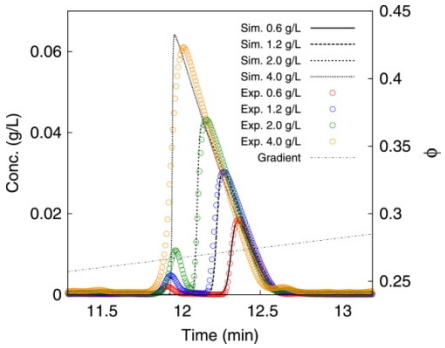

Figure 3: Comparison between experimental and simulated peaks in gradient conditions for four different crude peptide concentrations. The calculated peaks have been obtained using the model developed in this work and the Langmuir isotherm equation modified Vinj=5 µL reproduced with permissions from [21].

CONCLUSION

In this study, a model able to predict the peak profile in overloaded gradient conditions has been developed. The first step is the calculation of the adsorption isotherm under different isocratic conditions, using the Inverse Method. Then the variation of the adsorption isotherm parameters is correlated to the fraction of organic modifier in the mobile phase and, thus, to the gradient. The Inverse Method allows obtaining relevant thermodynamic information using just small amounts of product (some micrograms). The investigation of the thermodynamic equilibria involved in the retention of the peptide in RP-LC is a good starting point to develop a purification method through preparative chromatography in gradient conditions, avoiding the trial-and-error strategy. This would be particularly convenient especially for valuable compounds.

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