Elimination of the Sample Solvent Effect when Analysing Water Solutions of Basic Peptides by HILIC

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The Hydrophilic Interaction Chromatography (HILIC) separation mode offers many advantages for the analysis of basic peptides, such as good peak shape and separation efficiency, compared to, e.g., reversed-phase chromatography. However, the need to match the sample solvent with the highly organic mobile phase is limiting the use of HILIC. This article explores the suitability of a column-switch approach to enable the use of HILIC for large injection volumes of aqueous samples.

Introduction

The determination of potency and purity of the active pharmaceutical components is important in many stages of drug development, such as formulation development and stability studies for determination of shelf-life. Reversed-phase liquid chromatography (RPLC) is commonly used for this type of determinations. RPLC is a rugged and well-established technique for both small molecules and biomolecules. However, basic molecules, such as peptides containing the amino acids lysine or arginine, are difficult to analyse by RPLC, due to unwanted silanol interactions with the analyte, which can cause peak tailing and peak broadening [1].

An ion-pair reagent (such as TFA), or high amounts of salt in the mobile phase, can reduce such unwanted interactions, but can, on the other hand, cause problems if MS-detection is used. TFA causes ion suppression [2] and thus a decreased MS response, and non-volatile buffer salts are directly unsuitable to use in combination with MS.

Based on our experience, HILIC is an attractive technique for basic peptides since it provides good peak shape with MS-compatible mobile phases.

It is generally assumed that the retention in HILIC is mainly caused by partitioning of the analytes between the mobile phase and a water-enriched solvent layer close to the hydrophilic column surface [3,4]. This mechanism could explain the reduced secondary interactions in HILIC since the analytes are not interacting with the column material itself, or at least to a lesser extent, compared to RPLC.

What is limiting the use of HILIC is the need to match the sample solvent to the highly organic mobile phase. Since water is the strong solvent in HILIC, injection of aqueous solutions will lead to partial elution of the analytes at time of injection. This is called the sample solvent effect, which can lead to peak distortion and loss of retention and efficiency [5]. Thus, peptides formulated as aqueous solutions are not suitable for direct analysis by HILIC.

This article presents a fully automated way to eliminate the sample solvent effect also for large injection volumes of aqueous peptide samples in HILIC, by using a column-switch approach.

Experimental (HILIC method)

Acetonitrile (JT Baker, Ultra Gradient Grade) was used as HILIC mobile phase A. HILIC mobile phase B was prepared by adding 7.7 g ammonium acetate (Merck, p.a.) and 2000 µl glacial acetic acid (Merck, p.a.) to 1000 ml of water (measured pH 5.1). The trapping mobile phase was 5% acetonitrile in water.

[Lys^{8}]vasopressin and [Arg^{8}]vasopressin

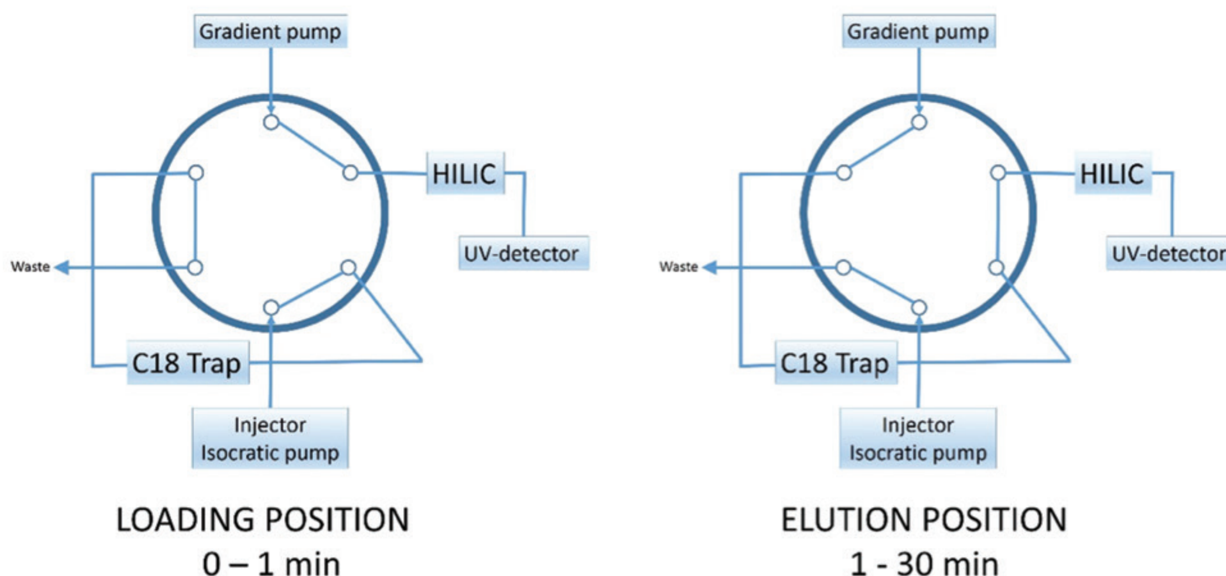

Figure 1. Configuration of the switching valve.

was purchased as lyophilised powder from Sigma-Aldrich and were dissolved in mobile phase B and mixed to contain 20 µg/ml of each component.

The experiment was performed on an Agilent 1260 HPLC with UV-detector, two pumps and one six port – two position switching valve, see Figure 1 for the instrument configuration. The trapping column was a 2.1x10 mm XTerra MS C_{18} precolumn (Waters). The HILIC column was an Accucore HILIC, 3.0 x 150 mm, 2.6 µm (Thermo Fischer Scientific). The column temperature was 60°C, and the detection wavelength was 277 nm. The injection volume was 100 µl.

The HILIC gradient is presented in Table 1. The flow of the trapping mobile phase was 1.0 ml/min.

Table 1: The HILIC gradient

HILIC gradient	%A	%B	Flow (ml/min)
0 min	85	15	0.8
1 min	85	15	0.8
20 min	62	38	0.8
21 min	85	15	0.8
30 min	85	15	0.8

For comparison the mixed vasopressin sample was also injected without using the column switching, i.e., the aqueous solution was injected directly on the HILIC column. All other method parameters remained the same.

Experimental (Reversed Phase method)

The same mobile phases, sample, and equipment were used in the reversed-phase experiment as in the HILIC experiment. The column switch was disconnected and the sample was injected directly on the column. The reversed-phase column was a Kinetex C_{18}, 3.0 x 150 mm, 2.6 µm (Phenomenex). All instrument settings were identical to the HILIC run, except the gradient and flow, see Table 2.

Table 2.

RP gradient	%A	%B	Flow (ml/min)
0 min	10	90	0.6
1 min	10	90	0.6
20 min	22	78	0.6
21 min	10	90	0.6
30 min	10	90	0.6

The reversed-phase method was not optimised for vasopressins and served only as a comparison to HILIC in terms of peak asymmetry and peak width with similar mobile phases.

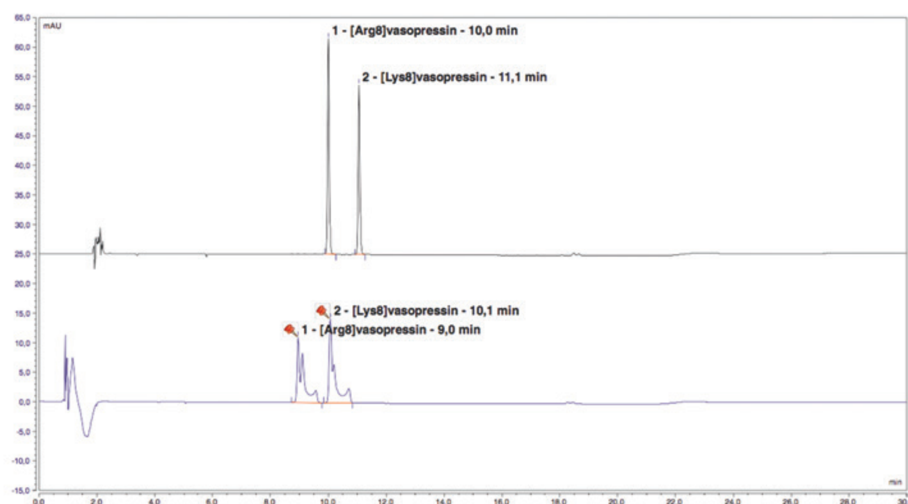

Figure 2: Overlay HILIC chromatograms of a) aqueous sample injected with the column switch approach (upper) and b) aqueous sample injected directly on the HILIC column (lower).

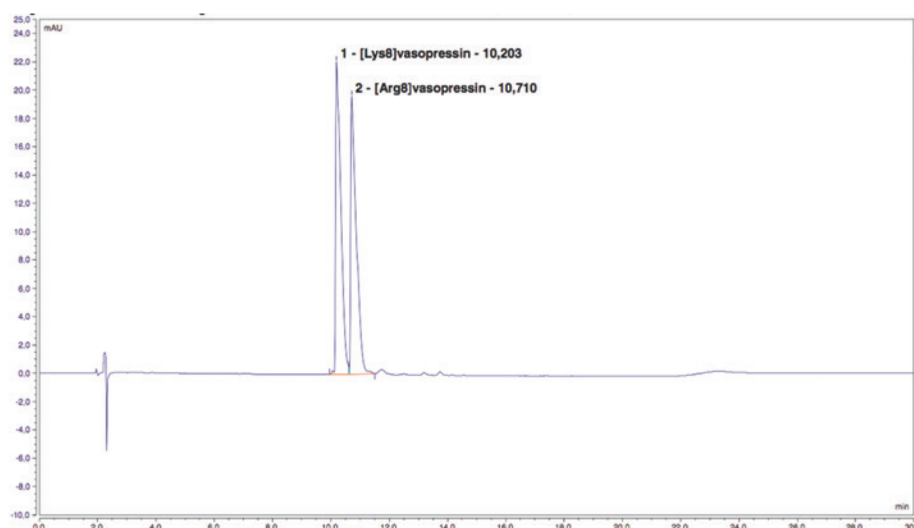

Figure 3: Reversed-phase chromatogram.

Results and Discussion

The HILIC and the reversed-phase chromatograms are presented in Figures 2 and 3, respectively, and the peak data comparison in Tables 3 and 4. The results show that HILIC is superior to reversed-phase chromatography for vasopressins in terms of peak width, asymmetry and resolution. In HILIC, the positively charged peptides elute as Gaussian peaks with an asymmetry factor of 1.0, whilst using MS-friendly mobile phases. In reversed-phase chromatography, the same peptides interact with the negatively charged residual silanol groups of the reversed phase bonded silica and show a high degree of tailing and peak broadening. Increased tailing and peak broadening decrease the peak capacity and the possibility to separate closely related impurities, which is observed by the decrease in resolution from 9.5 to 1.5 for HILIC and RPLC, respectively. It can be noted that the elution order of the two vasopressins is reversed in HILIC compared with reversed-phase, due to opposite column polarities.

Table 3. Peak characterisation data HILIC using column-switch.

Peak	Asymmetry factor (EP)	Resolution (EP)	Peak width (50% height)
[Arg^{8}]vasopressin	1.0	9.5	4 sec
[Lys^{8}]vasopressin	1.0	N/A	4 sec

Table 4. Peak characterisation data reversed-phase.

Peak	Asymmetry factor (EP)	Resolution (EP)	Peak width (50% height)
[Lys^{8}]vasopressin	3.3	1.5	11 sec
[Arg^{8}]vasopressin	3.4	N/A	13 sec

The chromatograms from injecting the aqueous sample in HILIC, with and without the column-switch installed, is compared in Figure 2. The results illustrate the problem of injecting an aqueous solution directly on to the HILIC column. With direct injection on to the column, the sample solvent effect causes severe peak splitting and peak broadening. However, when using the column switch approach, the sample solvent is removed before the HILIC column and thereby also the sample solvent effect.

The proposed setup with column switching and a trapping column works like an on-line solid-phase extraction method coupled with HILIC. Theoretically, there is no upper limit for the injection volume of aqueous sample since the sample is concentrated on the trap column and the sample solvent removed.

It should also be noted that the column-switching setup also de-salts the sample before being transferred to the HILIC column, since any buffer salts in the sample are not retained by the reversed-phase trap column and are sent to waste. Buffer salts are a potential problem in HILIC, since they are typically poorly soluble in high amounts of acetonitrile which could lead to precipitation and column blockage.

In summary, the presented technique provides a fully automated method to eliminate the sample solvent effect when analysing aqueous solutions by HILIC. A suggestion of a potential expansion of the principle can be to test different types of trap columns in combination with different mobile phases to explore selectivity and trapping.

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