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General method allowing the use of 100% aqueous loading conditions in reversed-phase liquid chromatography

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Abstract

Reversed-phase HPLC purification of peptides, using *n*-alkyl modified spherical silica, has become a widely used technique within the pharmaceutical industry. One drawback of these materials is the necessity of having at least 5% organic modifier in the mobile phase, in order to avoid de-wetting of the porous stationary phase.

For some preparative reversed-phase separations, it is an advantage if the feed solution can be loaded onto the column under 100% aqueous conditions.

This study describes the use of post-column pressure control to avoid de-wetting of regular reversed-phase stationary phases when operated under 100% aqueous conditions. The applicability of post-column pressure control as a means of maintaining the column fully wetted is demonstrated with various buffers and with packing materials having different alkyl-chain lengths.

Two peptides, insulin and oxytocin, in overloaded quantities, were loaded under 100% aqueous conditions onto a regular C8 column, and then eluted by a acetonitrile gradient following standard procedures. The retention volume and the peak shape showed that the separation was satisfactory, and proved that post-column pressure control can be used to overcome wettability problems, which are otherwise often observed for reversed-phase packing materials with high ligand density.

Keywords: Wettability; Aqueous loading conditions

1. Introduction

Reversed-phase HPLC purification of peptides has become a widely used technique within the pharmaceutical industry. The technique has proven to be an efficient and versatile separation tool, both for active pharmaceutical ingredients (APIs) and for intermediate building blocks. Stationary phases based on alkyl bonded spherical silica are most often used, since such materials have a reproducible, uniform pore structure, and are chemically and mechanically stable [1]. However, such materials cannot be used under two conditions: first, under alkaline pH, the silica backbone undergoes gradual dissolution [2]; and secondly, the use of mobile phases with a low content of organic modifier (<5%) generally leads to what is known as "phase collapse" [3,4]. The latter is best described as a de-wetting phenomenon, in which the highly aqueous mobile phase is excluded from the hydrophobic pore system due to surface tension. The loss of wetted surface results in a decrease in accessible interaction sites for the solute, and leads to a loss in retention [5]. The process can easily be reversed by purging the packed bed with a high content of organic modifier, and the application of post-column pressure is known to assist the process [4,6].

The problem of mobile-phase expulsion becomes apparent when separating highly polar solutes, which are only retained under 100% aqueous conditions and where highly aqueous mobile phases must be chosen for solubility reasons [7]. Parameters that affect the degree of de-wetting are well described [5,6], and many manufacturers of HPLC packing material have addressed the problem by introducing different types of "wettable" reversed-phase stationary phases. Wettability, and hence reproducible retention times under 100% aqueous mobile-phase conditions, can be maintained by using either non-endcapped or polar endcapped bonded phases [5,6], or by using specially designed packing materials with polar embedded alkyl chains [7]. The use of long-chain (C_{30}) alkyl phases or wide-pore silica also enables the use of highly aqueous mobile phases, and packing

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materials with a low ligand density are also known to be fairly immune to de-wetting [7].

Although the problem is well described and products that circumvent the risk of mobile-phase expulsion are on the market, the focus of all investigations appears to be on analytical HPLC applications. To our knowledge, no preparative applications in which one of the specially designed stationary phases was employed has been reported, nor are we aware of work describing the use of regular stationary phases under accommodated conditions. A possible reason is the reduced hydrolytic stability of wettable phases [5,8]. Although peptides are most often separated by gradient elution in which the content of the organic modifier is in a range where the hydrophobic pore system is fully wetted, the need to subject the reversed-phase packing to 100% aqueous conditions may arise during the loading step. For many preparative reversed-phase separations, it is an advantage if the crude feed solution can be loaded onto the column under 100% aqueous conditions. This enables direct downstream processing from previous purification steps, such as ion exchange, without additional desalting, dilution or concentration steps.

The objective of this study was to investigate the beneficial effect of post-column pressure on the wettability of regular reversed-phase stationary phases (C_{18}, C_8, C_4, C_1). Although pressure is mentioned as a parameter reducing the degree of phase collapse, and as an aid for regenerating collapsed phases [6], its potential as a process parameter in preparative chromatography has not been previously described. We describe the successful use of post-column pressure under 100% aqueous loading conditions for a non-linear chromatographic separations of two peptides, insulin and oxytocin. Further, we have determined the minimal pressure required for several stationary phases and for different buffer systems.

2. Experimental

2.1. Apparatus and reagents

All chromatographic experiments were performed with a Waters 600E HPLC pump equipped with a Waters 486 UV detector (Waters, Milford, MA, USA). The post-column pressure regulation valve was purchased from Alltech (Deerfield, IL, USA).

Buffers were prepared from de-ionized water from a MILLI-Q purification system (Millipore, Milford, MA, USA) and were filtered through a 0.4 μm filter before use. Potassium dihydrogen phosphate and ammonium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany), ammonium acetate and trifluoroacetic acid (TFA) from Fluka (Buchs, Switzerland). Acetonitrile was bought from Lab-Scan (Dublin, Ireland). All columns were 4.6 (i.d.) mm × 250 mm and were obtained from Eka Chemicals AB, Bohus, Sweden. The columns used for this study were KR100-10-C1, KR100-10-C4, KR100-10-C8, KR100-10-C18 (ligand density: 4.0 (C1), 3.8 (C4), 3.7 (C8), 3.5 (C18) μmol/m^2).

Guanine, propyl benzene, human insulin and oxytocin were bought from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Determination of retention volumes

Columns were first purged with five column volumes of acetonitrile/water (70:30) without pressurizing the column at its outlet. The columns were then equilibrated with 100% aqueous buffer for 40 min at 0.5 ml/min, after the pressure drop at the column inlet had been adjusted by means of the post-column reduction valve. The pressure was set by monitoring the obtained back pressure (23 bar) with acetonitrile/water (70:30), without any post-column pressure. A theoretical value for a 100% aqueous mobile phase was calculated by taking the viscosity into account ($O_{\text{water/ACN (30:70)}} = 0.82$ cP and $O_{\text{water}} = 0.1$ cP), giving a value of 28 bar. In order to obtain a column back pressure of, e.g. 50 bar, the reduction valve was adjusted in such a way that 78 bar pressure drop were measured at the column inlet. Once the equilibration had been completed, 2 μl of the sample solution (5 mg/ml mobile phase) was injected and the chromatogram was recorded at 254 nm. The pressure was then reduced for the next injection and the procedure was repeated, omitting the initial purging step with acetonitrile/water (70:30). For one buffer, KH_2PO_4, the post-column pressure was stepwise increased back to 95 bar in order to see if the de-wetting of the hydrophobic phase was reversible.

2.3. Aqueous loading conditions for peptide separations

2.3.1. Insulin separations

A KR100-10-C8 column (4.6 mm × 250 mm, packing density: 0.6 g/ml) was equilibrated with 10 column volumes of the 100% aqueous mobile phase (water + 0.1% TFA or 50 mM ammonium acetate at pH 4.5) at 0.5 ml/min, with a post-column pressure of 25 bar, and then 500 μl of human insulin solution (10 mg human insulin/ml water + 0.1% TFA or 10 mg human insulin/ml + 0.15% acetic acid) was injected. Such an injection corresponds to a relative loading of 2 mg/$g_{\text{packing material}}$. The gradient elution is shown in Table 1. Chromatograms were recorded at 280 nm.

2.3.2. Oxytocin separation

The separation was conducted according to the procedure described in Section 2.3.1, with a mobile-phase system consisting of water/acetonitrile + 0.1% TFA. The injection solution was prepared by dissolving 10 mg oxytocin in 1 ml water + 0.1% TFA. Five hundred microliters of this sample solution was injected, and the peptide was separated by applying the gradients shown in Table 2. The relative load of this separation was 2 mg/$g_{\text{packing material}}$.

Table 1
Gradients used for the overloaded separation of insulin: (A) for loading under 100% aqueous conditions and (B) as a control separation, the column having been equilibrated with 10% acetonitrile prior to the injection

t (min)	Flow (ml/min)	Acetonitrile (%)
(A) 100% aqueous loading conditions		
0	0.5	0 (0)
15	0.5	0 (0)
15.1	0.5	10 (10)
30	0.5	10 (10)
30.1	0.5	25 (26)
110.1	0.5	33 (34)
140	0.5	33 (34)
(B) Control		
0	0.5	10 (10)
15	0.5	10 (10)
15.1	0.5	25 (26)
95.1	0.5	33 (34)
140	0.5	33 (34)

Values in parentheses refer to the separation with ammonium acetate as buffer. Mobile phase A consisted of water + 0.1% TFA, or 50 mM ammonium acetate at pH 4.5, and mobile phase B consisted of acetonitrile.

Table 2
Gradients used for the overloaded separation of oxytocin: (A) for loading under 100% aqueous conditions and (B) as a control separation, the column having been equilibrated with 10% acetonitrile before the injection

t (min)	Flow (ml/min)	Acetonitrile (%)
(A) 100% aqueous loading conditions		
0	0.5	0
15	0.5	0
15.1	0.5	10
30	0.5	10
30.1	0.5	16
110.1	0.5	24
140	0.5	70
(B) Control		
0	0.5	10
15	0.5	10
15.1	0.5	16
95.1	0.5	24
140	0.5	70

3. Results and discussion

Two approaches were chosen in order to demonstrate that controlled post-column pressure can be used during the actual chromatographic separations and during the loading step, and not only to assist column reconditioning [6]. First, the utility of the regular reversed-phase columns under 100% aqueous mobile-phase conditions was tested by measuring the retention volume of guanine as a function of post-column pressure, type of buffer and length of alkyl chains of the surface modified packing material. Secondly, two peptides, insulin and oxytocin, were injected onto the chromatography column under moderately overloaded, non-linear conditions ($2\,mg/g_{packing\,material}$), and the retention volume and peak profile were compared between a loading step performed under 100% buffered conditions and one performed with 10% acetonitrile present. Although these experiments were carried out using analytical columns (4.6 mm × 250 mm), the results are applicable for larger column dimensions, since scale-up in chromatography is largely a matter of preserving thermodynamic parameters [9,10]. An overloaded injection of $2\,mg/g_{packing\,material}$, clearly represents the non-linear part of the adsorption isotherm, due to the triangular peak shape, hence indicating the feasibility of the procedure for preparative applications. An analytical injection (not shown) leads to a narrow, symmetrical peak eluting at the same retention time as the back part of the peak of the overloaded injection.

3.1. Determination of the required post-column pressure

The effect of post-column pressure on the retention volume of a polar solute, guanine, was determined under 100% aqueous conditions. A KR100-10-C8 column was equilibrated with 20 mM KH_2PO_4 buffer with an initial post-column pressure of 82 bar. The elution volume of guanine was measured while the post-column pressure was gradually reduced. Fig. 1 (insertion) shows that the elution volume was nearly constant between 82 and 25 bar. When the post-column pressure was reduced further, the elution volume dropped rapidly.

This behavior is explained by the Washburn Eq. (1). Pressures (220–290 bar) required to wet and fill a pore with water can be calculated when assuming largest possible contact angles (i.e. 140°–180°) between water and a 100 Å pore. However, with a contact angle of 95°, a lower wetting pressure of 25 bar will be sufficient.

$$\Delta P = \frac{4\gamma \cos\theta}{d} \quad (1)$$

where P is the intrusion pressure (J/m^3), K the surface tension (J/m^2), θ the contact angle (°), and d the pore diameter (m).

Raising the pressure back to 95 bar (the maximum value used in the experiments) did not re-wet the pore system, and the retention time of guanine remained the same as that obtained without any post-column pressure. The resulting hysteresis can be explained by the underlying contact angle hysteresis, which is known to be significant for geometrically heterogeneous surfaces. The differences between the advancing and the receding contact angles may be as much as 50° [11].

We conclude that the regular C8 phase can be operated under 100% aqueous conditions at post-column pressures as low as 25 bar. In order to establish if these findings are valid for other buffers than the potassium dihydrogen phosphate buffer, three additional buffer systems were tested under identical conditions.

As can be seen in Fig. 1, the choice of buffer does not influence the post-column pressure required in order to keep the pore system fully wetted. In all cases, the

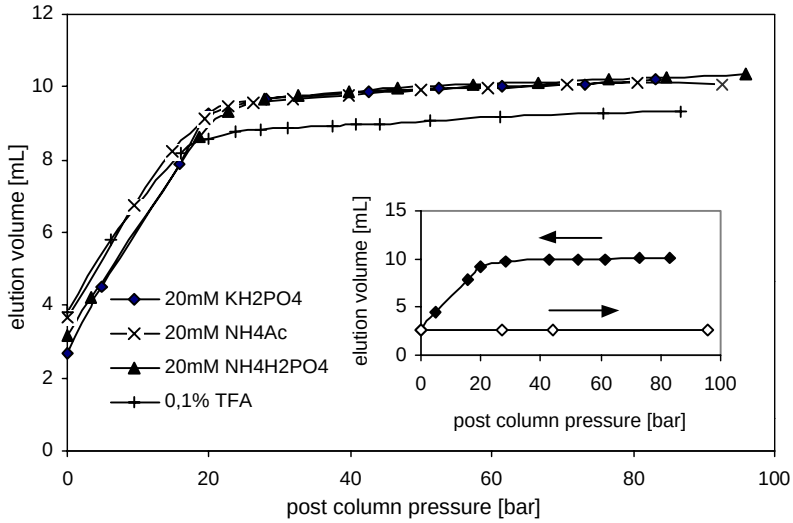

Fig. 1. Effect of post-column pressure on the elution volume of guanine. Stationary phase: KR100-10-C8, 4.6 mm × 250 mm. Mobile phase: 20 mM KH_2PO_4 at pH 4.8 (◆), 20 mM NH_4Ac at pH 4.8 (×), 20 mM $NH_4H_2PO_4$ at pH 4.8 (▲), 0.1% TFA (+). Flow rate: 0.5 ml/min. Descending post-column pressure in all cases. Inset: Elution volume of guanine as a function of post-column pressure. Stationary phase: KR100-10-C8, 4.6 mm × 250 mm. Mobile phase: 20 mM KH_2PO_4 at pH 4.8. Flow rate: 0.5 ml/min. Solid symbols represent descending post-column pressure and open symbols represent ascending post-column pressure.

mobile-phase expulsion commences below 25 bar. Guanine exhibits a lower elution volume with TFA than it does with the phosphate- and acetate-buffered systems. We suggest that this can be attributed to the lower pH when 0.1% TFA is used, compared with the value of 4.8 when the other buffers are used.

3.2. General effect of post-column pressure on retention

The experiments described above clearly show that reversed-phase columns with a high ligand density can be operated under 100% aqueous conditions when applying a post-column pressure of at least 25 bar. However, a slight shift in elution volume was observed for all buffer types at pressure values that were sufficiently high to maintain the hydrophobic porous system fully wetted. For the C8 column, a nearly linear decrease in elution volume of 10 μl/bar was measured. The question arose whether the accessible surface area was reduced gradually due to mobile-phase expulsion over the entire pressure range, or if the observed shift could be attributed to the applied pressure solely. In the latter case, the shift will be independent of the de-wetting phenomenon. In order to answer this question, an experiment, similar to the one described above, was conducted with a mobile phase for which full wettability of the reversed-phase column could be assured. The elution volume of propyl benzene was monitored over the same range of post-column pressure, but with a mobile phase containing 70% acetonitrile (Fig. 2).

The results showed that the elution volume also shifts under regular reversed-phase conditions, where full access of the mobile phase to the pore system can be ensured.

3.3. Reproducibility

When measuring the effect of post-column pressure on the elution volume of guanine (Fig. 1), it became apparent that the mobile-phase expulsion that occurs at post-column pressures below 25 bar is not reversible when the pressure is subsequently increased. Although the results showed that the regular reversed-phase packing materials should not be operated under this critical post-column pressure, it is possible that the small shift in retention time observed above 25 bar is also irreversible. In this case, the original retention times will not be reached upon adjusting the post-column pressure to a value where a separation was previously achieved, and chromatography thus becomes irreproducible due to a gradual decline in the accessible surface area. In the case of a preparative separation, where the feed is repeatedly charged under 100% aqueous conditions, a continuous decline in accessible surface will result in a broader loading band due to reduced capacity, and this will impair the purification.

Measuring the elution volume of guanine while changing the post-column pressure in cycles from 75 to 55 to 35 bar and back to 55 and 75 bar allowed us to conclude that the original retention times can be re-achieved. Fig. 2 (inset) shows that the elution volumes determined in the second and third cycles are not generally lower than those measured during the first cycle.

3.4. Effect of surface modification type

While C18 showed a pressure dependency similar to the one previously seen for C8, both C4 and C1 remained almost unaffected by post-column pressures below 20 bar

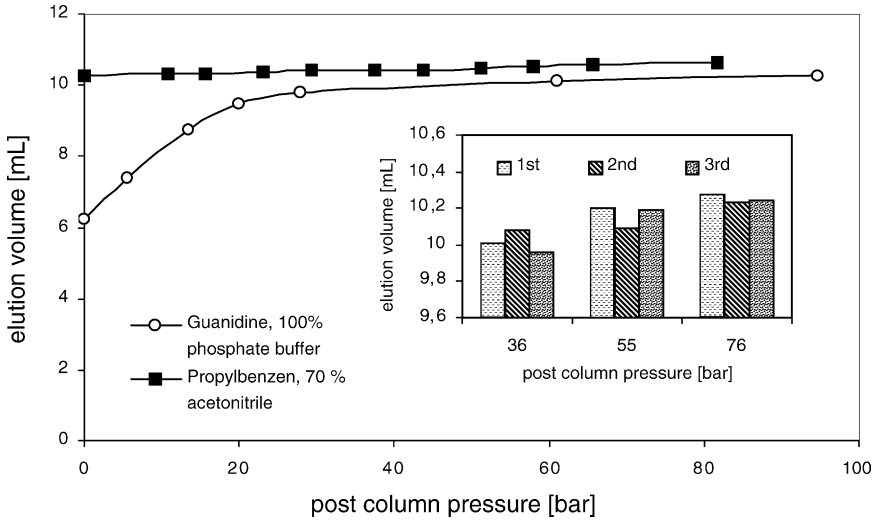

Fig. 2. Effect of post-column pressure on the elution volume under regular reversed-phase HPLC conditions. Stationary phase: KR100-10-C8, 4.6 mm × 250 mm. Flow rate: 1 ml/min. The elution volume of propyl benzene was determined with acetonitrile/water (70:30) as mobile phase. For comparison reasons, guanine was run with 20 mM KH_2PO_4 at pH 4.8 as mobile phase. Inset: Effect of alternating post-column pressure on the reproducibility of the elution volume. Stationary phase: KR100-10-C8. Mobile phase: 20 mM NH_4Ac at pH 4.8. Flow rate: 0.5 ml/min.

(Fig. 3). These findings are well in agreement with the behavior mentioned in literature [7]. The robustness of the short-chain modified packing materials can be explained by the lack of conformational freedom and a retention mechanism based on adsorption rather than on partitioning [12]. The change in retention mechanism can be deduced from the elution volumes of guanine measured for the different columns.

The elution volume decreased from C18 to C8 due to a decrease in the hydrophobic character. However, this trend did not continue for C4- or C1-modified packing materials. While C4 showed a retention time similar to that of C8, guanine was retained significantly longer on C1 than on C4 and C8 columns. We suggest that the retention mechanism changes between C8 and C4. For the short-chain (C1 and C4) phases, retention is predominantly based on adsorption, where electrostatic interactions with the residual silanol groups of the silica matrix gain in importance with the decrease in alkyl-chain length.

3.5. Peptide separations

In order to show that post-column pressure can be used to facilitate 100% aqueous loading conditions for preparative peptide separations, two peptides, oxytocin (nine amino acids) and insulin (51 amino acids), were used as test samples. The retention times and peak widths of the peptides were compared between 100% aqueous loading conditions

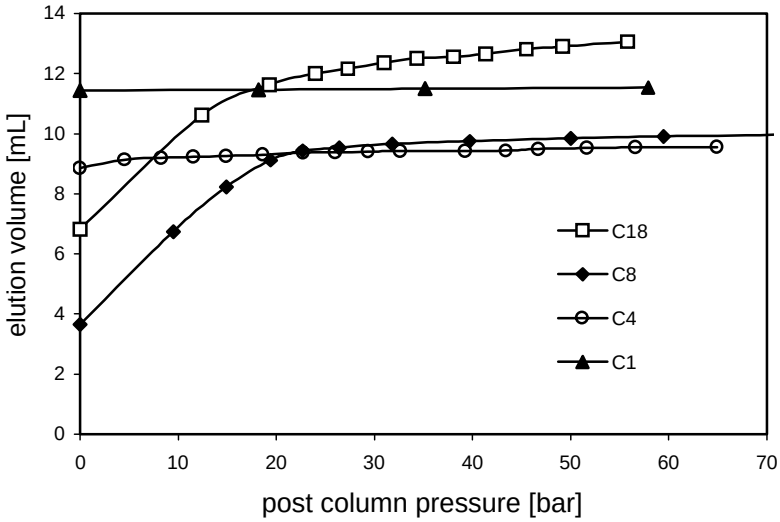

Fig. 3. Effect of post-column pressure on the elution volume of guanine for different types of surface modified packing materials. Mobile phase: NH_4Ac at pH 4.8. Set flow rate: 0.5 ml/min.

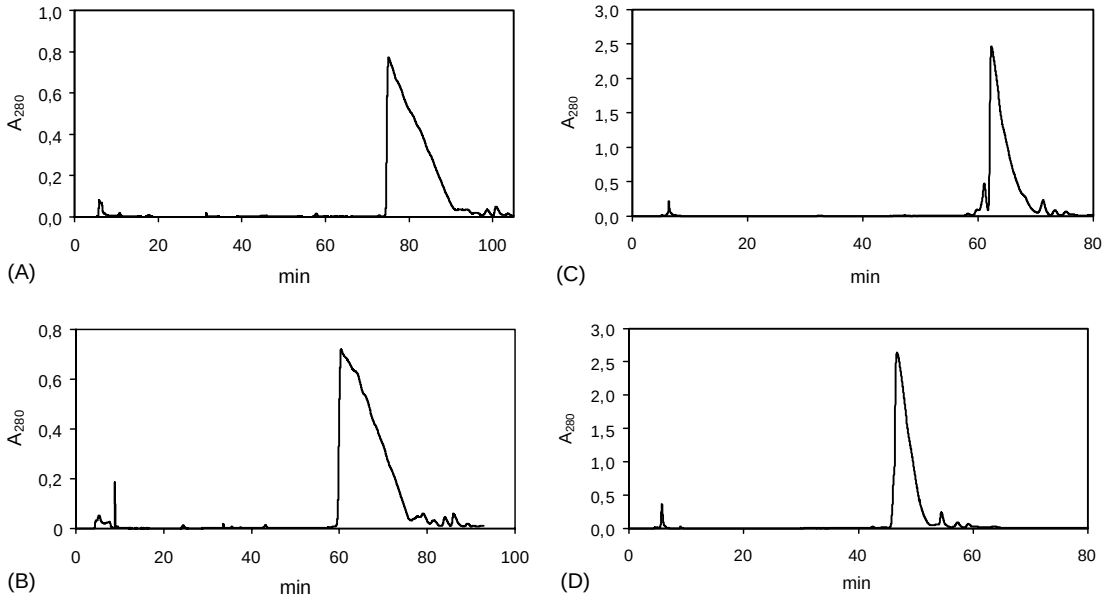

Fig. 4. Chromatograms obtained from a 5 mg injection of insulin. (A) Loading in water + 0.1% TFA and (B) loading in water/acetonitrile (90:10) + 0.1% TFA. Stationary phase: KR100-10-C8 (4.6 mm × 250 mm). Mobile phase A: water + 0.1% TFA and mobile phase B: acetonitrile. Gradient: 25–33% acetonitrile/80 min. (C) Loading in 50 mM NH_4Ac at pH 4.5 and (D) loading in 50 mM NH_4Ac/acetonitrile (90:10) at pH 4.5. Mobile phase A: 50 mM NH_4Ac at pH 4.5 and mobile phase B: acetonitrile. Gradient: 26–34% acetonitrile/65 min. Flow rate in all cases: 0.5 ml/min.

and a control separation in which the column was equilibrated with 10% acetonitrile. In the case of the 100% aqueous conditions, a 25 bar post-column pressure was applied. As can be seen, the moderately overloaded preparative injection of insulin was well retained on the C8 column when loaded under 100% aqueous conditions. The difference in retention times between chromatogram A and B in Fig. 4 can be explained by an extra isocratic concentration step in separation A. Before the gradient was started, the column was purged 15 min with water + 0.1% TFA, followed by another 15 min wash with water/acetonitrile (90:10) + 0.1% TFA. When recording the control chromatogram B, the column was only washed for 15 min with water + 0.1% TFA/acetonitrile (90:10). The retention times were altered by only 4 min. In order to show that post-column pressure can be used in general to circumvent wettability problems during a loading step, ammonium acetate was tested as an alternative mobile-phase buffer. Insulin was separated under the same conditions as those described above, and the peptide was again fully retained under 100% aqueous loading conditions. Moreover, an impurity that co-eluted with the insulin peak when the loading was performed in 10% acetonitrile could be separated entirely (Fig. 4, chromatograms C and D).

The experiment was repeated with oxytocin in order to confirm that the proposed procedure works for a broad range of peptides. Fig. 5 shows that the smaller peptide was also well retained under 100% aqueous conditions.

Both retention times and peak widths of the chromatograms of peptides loaded under 100% aqueous loading resemble those of chromatograms obtained under regular conditions. The fact that this is the case for overloaded injections, representing the non-linear part of the adsorption isotherm, shows that the adsorption capacity of the stationary phase is not impaired by the use of a 100% aqueous mobile phase.

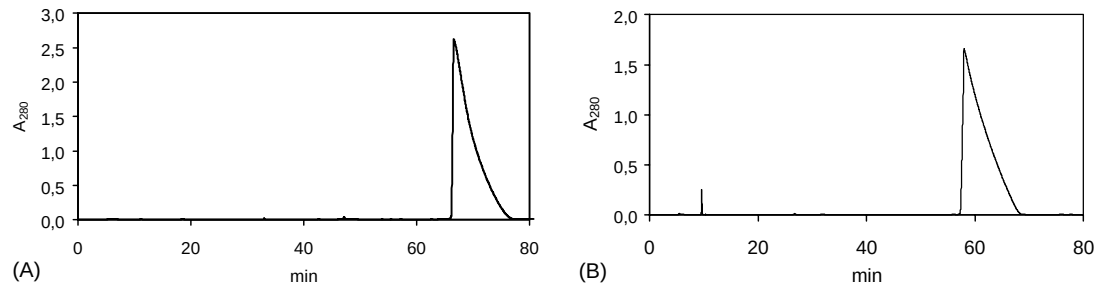

Fig. 5. Chromatograms obtained from a 5 mg injection of oxytocin. (A) Loading in water + 0.1% TFA and (B) loading in water/acetonitrile (90:10) + 0.1% TFA. Stationary phase: KR100-10-C8 (4.6 mm × 250 mm). Mobile phase A: water + 0.1% TFA and mobile phase B: acetonitrile. Gradient: 25–33% acetonitrile/80 min. Flow rate: 0.5 ml/min.

4. Conclusions

Densely bonded, regular reversed-phase stationary phases, known to undergo phase collapse when used under 100% aqueous conditions, functioned properly when the post-column pressure was kept above 25 bar. The observation that a phase that has collapsed cannot be re-wetted at 25 bar (Fig. 1, inset) was explained by the difference in the contact angle between advancing and receding wetting movements for geometrically heterogeneous surfaces of porous packing material.

The retention time for guanine was constant above 25 bar. The retention time dropped as the pressure was reduced below 25 bar, as a consequence of the lower accessible surface area. We conclude that the post-column pressure prevents mobile-phase expulsion from the pore system, the adsorption capacity thus remains unimpaired and the method seems adequate for loading steps under 100% aqueous conditions. This enables direct downstream processing from previous steps, such as ion exchange, in a purification process without requiring additional desalting, dilution or concentration steps. This makes the method attractive from a process economic point of view. A schematic structure of a preparative HPLC system adjusted for the application of post-column pressure is illustrated in Fig. 6.

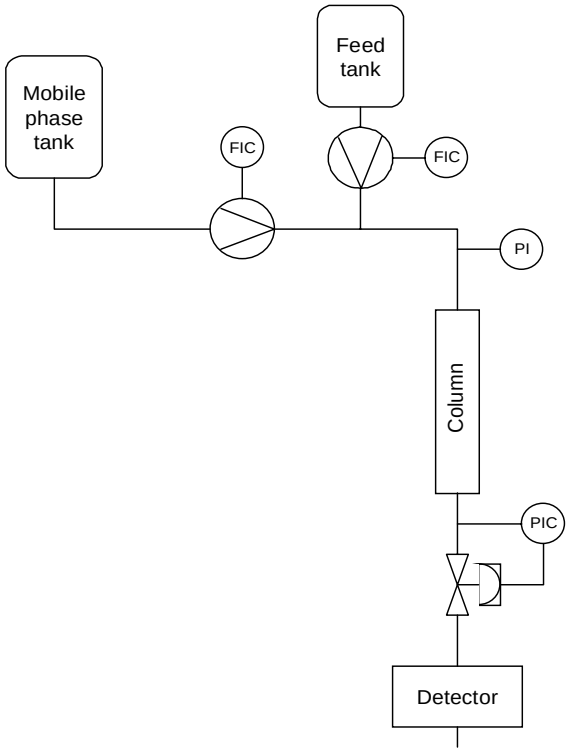

Fig. 6. Basic flow chart of an HPLC system equipped with a post-column reduction valve.

Although the pressure rating of a column system is often the limiting factor in preparative HPLC, a post-column pressure of 25 bar is still feasible. For a column packed with particles of diameter 10 μm and a bed length of 25 cm, the linear flow rate can be up to 6.3 cm/min in order to have a resulting pressure drop below 60 bar (mobile-phase viscosity $O = 1$ cP). In order for a 25 bar post-column pressure to be applied, the maximum linear flow rate must be reduced to 3.6 cm/min in order to fulfill the pressure limitations. Since the post-column pressure of 25 bar is only necessary during the equilibration, loading and concentration step, the additional limitation of the maximum flow rate does not impair productivity during the actual gradient elution.

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